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GRAINS AND IRRITABLE BOWEL SYNDROME

Randomised controlled trials with low FODMAP rye and wheat bread

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ACADEMIC DISSERTATION

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III Pirkola L, Laatikainen R, Loponen J, Hongisto S-M, Hillilä M, Nuora A, Baoru Y, Linderborg KM, Korpela R and Freese R. Low-FODMAP vs. regular rye bread in irritable bowel syndrome: randomized SmartPill® study. World Journal of Gastroenterology 2018; 24: 1181-1284.

IV Laatikainen R, Jalanka J, Loponen J, Hongisto S-M, Hillilä M, Koskenpato J, Korpela R, Salonen A. Randomised Clinical Trial: Effect of low-FODMAP rye bread versus regular rye bread on the intestinal microbiota of irritable bowel syndrome patients: Association to individual symptom variation. BMC Nutrition 2018, submitted.

The publications are referred to in the text by their Roman numerals. The original articles are reprinted with kind permission of the publishers.

MAIN ABBREVIATIONS

ANOVA	Analysis of Variance
AXOS	Arabinoxylooligosaccharide
ATI	Amylase Trypsin Inhibitor
CI	Confidence Interval
CD	Coeliac Disease
CNS	Central Nervous System
CRO	C-Reactive Protein
ENS	Enteric Nervous System
FABP2	Fatty Acid-Binding Protein 2
FD	Functional Dyspepsia
FODMAPs	Fermentable Oligo-, Di-, Monosaccharides And Polyols
FOS	Fructooligosaccharide
FOXP3	Forkhead Box P3
FBDSI	Functional Bowel Disorders Severity Index
GOS	Galacto-Oligosaccharide
GWAS	Genome Wide Association Scan
GERD	Gastro-Esophageal Reflux Disease
GFD	Gluten Free Diet
GI	Gastrointestinal
GSRS	Gastrointestinal Symptom Rating Scale
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-C	IBS with Constipation
IBS-D	IBS with Diarrhoea
IBS-M	Mixed IBS
IBS-SSS	Symptom Severity Score, patient-rated intensity of IBS
IBS QoL	Quality of Life questionnaire designed for studies in IBS
IBS-U	Unspecified IBS (no major motility disorder present)
IL	Interleukin
LBP	Lipopolysaccharide Binding Protein
miRNA	Micro-Ribonucleic Acid
NCGS	Non-Coeliac Gluten Sensitivity
NCWS	Non-Coeliac Wheat Sensitivity
NNT	Number Needed to Treat
OUT	Operational Taxonomic Unit
QoL	Quality of Life
PCoA	Principal Coordinates Analysis
PI-IBS	Post-infectious Irritable Bowel Syndrome
RBB	Repeated Beat Beating
RCT	Randomized Controlled Trial

SCD	Specific Carbohydrate Diet
SD	Standard Deviation
SIBO	Small Intestinal Bacterial Overgrowth
TLR	Toll Like Receptor
VAS	Visual Analog Scale

ABSTRACT

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder, in global terms affecting more than every tenth person. Grains are a staple food and an integral part of daily diets all around the world. The FODMAPs (Fermentable Oligo-, Di-, Monosaccharides And Polyols), gluten and amylase trypsin inhibitors (ATIs), which are present in wheat, barley and rye, might be at least part of the reason why gluten-containing grains are often considered as triggers of functional gastrointestinal symptoms. However, the relative contributions and comparative importance of these substances in symptom aggravation remain unclear. The main purpose of this thesis was to determine whether a reduction in the levels of FODMAPs and ATIs in grain products would improve gastrointestinal tolerability of rye and wheat in IBS and non-coeliac gluten sensitivity (NCGS).

The intervention grain products used in the studies were produced by sourdough baking method which resulted in a reduced FODMAP and ATI content in the test breads. Four different randomised studies were performed among a female dominant adult population suffering from IBS.

In study I, subjects with IBS (n=87) consumed either low FODMAP rye bread or regular rye bread for 4 weeks. This cross-over trial revealed that the low FODMAP rye bread reduced colonic gas formation and alleviated some IBS symptoms (flatulence, abdominal pain, cramps and borgorygmi) but that there was no difference between the breads in overall symptom control or quality of life.

In the second study, 26 IBS subjects with poor subjective tolerance for wheat were randomised to either wheat bread with yeast as a leavening agent or wheat bread made by a sourdough baking method which was free of additives and had a lower content of ATIs and FODMAPs. No difference was detected in the gastrointestinal tolerance of these two breads during the observation period of seven days.

In study III, the effects of low FODMAP rye bread and regular rye bread on intestinal events were evaluated with a wireless motility capsule (SmartPill®, Given Imaging ltd, Israel) in seven subjects with IBS. It was found that a low FODMAP content reduced colonic fermentation as measured by breath hydrogen excretion but there were no differences between the bread periods in intestinal pH, transit time, pressure or contractions. However, the overall symptom severity and the total score of symptoms, evaluated with a visual analog scale, were associated with colonic pressure during the period when the subjects consumed the regular bread.

Study IV was performed as a sub-study of study I by examining microbial changes in feces being analysed in 50 subjects. The low FODMAP rye bread reduced the abundance of the genus Klebsiella in fecal samples in comparison to regular rye bread but no other

statistically significant changes in fecal microbiota were found between the periods when the test subjects ate the regular and low-FODMAP rye breads.

The results emerging from this thesis demonstrated that the rye bread with the low FODMAP content reduced colonic fermentation and therefore there was less gas formation when compared to the consumption of regular bread. It can be speculated that low FODMAP breads may represent a means of improving tolerability of breads among subjects with IBS but switching from regular to a low FODMAP version of bread is likely to exert only modest effects on objectively measured transit times, intestinal pH, intraluminal pressure and faecal microbiota. Low FODMAP breads can be used as a part of a holistic low FODMAP diet for individuals with IBS.

TIIVISTELMÄ

Ärtyvän suolen oireyhtymä (IBS) on yleinen mahasuolikanavan vaiva, sen esiintyvyys on maailmanlaajuisesti noin 11 %. Viljatuotteet ovat laajasti käytettyjä elintarvikkeita ympäri maailman ja siten tärkeä osa ruokavaliotamme. Rukiin, vehnän ja ohran FODMAP-hiilihydraatit (Fermentable Oligo-, Di-, Monosaccharides And Polyols), gluteeni ja amylaasi-trypsiini inhibittorit (ATI:t) voivat aiheuttaa oireita toiminnallisissa vatsavaivoissa kuten IBS:ssä. Ei ole kuitenkaan tarkkaa tietoa siitä, mikä on näiden tekijöiden suhteellinen tärkeys toisiinsa verrattuna oireiden aiheuttajana. Tämän väitöskirjan tavoitteena oli tutkia lievittääkö FODMAP-hiilihydraattien ja ATI:n vähentäminen ruis- ja vehnätuotteissa niiden siedettävyyttä henkilöillä, joilla on ärtyvän suolen oireyhtymä tai gluteeniherkkyys.

Tässä väitöskirjatutkimuksessa tutkittiin erityisten hapatusmenetelmien avulla valmistettuja ruis-ja vehnäleipiä, joiden FODMAP- ja ATI-pitoisuudet olivat vähäisemmät kuin tavanomaisissa ruis- tai vehnäleivissä. Väitöstutkimukseen sisältyy neljä erillistä satunnaistettua tutkimusta.

Ensimmäiseen vaihtovuorotutkimukseen osallistui 87 henkilöä, joille syötettiin joko vain vähän FODMAP-hiilihydraatteja sisältävää tai tavanomaista ruisleipää kumpaakin 4 viikon ajan. Tutkimuksessa havaittiin, että vähemmän FODMAP-hiilihydraatteja sisältävä leipä aiheutti vähemmän kaasun kertymistä suolistoon ja samalla vähensi ilmavaivojen tunnetta, vatsakipua, vatsan kurinaa ja kramppeja. Elämän laadussa tai IBS-SSS mittarilla mitatussa kokonaisoirekuvassa ei kuitenkaan havaittu eroja leipäjaksojen välillä.

Toisessa tutkimuksessa, johon osallistui 26 aikuista, tutkittiin hiivalla kohotetun, lisättyä gluteenia ja lisäaineita sisältävän tavanomaisen vehnäleivän ja hapattamalla tehdyn lisäaineettoman leivän siedettävyyttä. Hapatetussa vehnäleivässä oli vähemmän ATI:a ja FODMAP-hiilihydraatteja, mutta mahasuolikanavan siedettävyydessä ei havaittu eroja ryhmien välillä viikon koejaksolla.

Kolmas tutkimus vertaili vain vähän FODMAP-hiilihydraatteja sisältävän ja tavanomaisen ruisleivän suolistovaikutuksia yhden vuorokauden ateriakokeessa, jossa oireiden lisäksi tutkittavat myös nielivät erityisen langattoman suoliston olosuhteita mittaavan kapselilaitteen (SmartPill®, Given Imaging ltd, Israel) mahasuolikanavan toimintojen, erityisesti pH:n, läpikulkuajan, suoliston sisäiseen paineen ja supistusten monitoroimiseksi. Oireiden vaikeusaste liittyi paksusuolen sisäiseen paineeseen tavanomaista ruisleipää sisältäneen koepäivän aikana, mutta leipäjaksojen välillä ei havaittu eroja mitatuissa suureissa. Oireiden vaikeusastetta arvioitiin IBS-oireiden summana ns. visual analog scale -mittarilla.

Neljännessä tutkimuksessa analysoitiin ensimmäiseen tutkimukseen osallistuneiden 50 tutkittavan mikrobisto ulostenäytteistä ja oirekehitystä neljän viikon aikana seurattiin. Tutkimuksessa havaittiin, että vain vähän FODMAP-hiilihydraatteja sisältävän

ruisleipäjakson aikana Klebsiella bakteeria esiintyi vähemmän ulostenäytteissä kuin tavanomaista ruisleipää sisältäneen koejakson aikana. Muita eroja suoliston mikrobistossa ryhmien välillä ei havaittu.

Tämän tutkimussarjan mukaan vain vähän FODMAP-hiilihydraatteja sisältävä ruisleipä vähentää paksusuolessa tapahtuvaa käymisreaktiota (fermentaatiota) ja siten kaasun tuotto suolistossa vähenee. Edelleen väitöstutkimuksen mukaan vähemmän FODMAP:ja sisältävät leivät voivat olla paremmin siedettyjä kuin tavanomaiset leivät, mutta tavanomaisen leivän vaihtaminen vain vähän FODMAP-hiilihydraatteja sisältävään leipään ei riitä yksistään aiheuttamaan kovin merkittäviä eroja mahasuolikanavan pH:ssa, läpikulkuajassa, paineessa tai mikrobistossa. Vain vähän FODMAP-hiilihydraatteja sisältävää leipää voidaan käyttää osana kokonaisvaltaista FODMAP-hiilihydraattien rajoitusta ärtyvän suolen oireyhtymässä.

1 INTRODUCTION

The consequences of defective digestive processes are reflected in many historical proverbs; *Hippocrates*, the father of medicine, is quoted as saying, "Bad digestion is at the root of all evil", the French philosopher *Voltaire*, "The fate of a nation has often depended upon the good or bad digestion of a prime minister, and the poet *William Shakespeare*, "Things sweet to taste prove in digestion sour." The scientifically documented history of existence of irritable bowel syndrome dates back to at least the 17th century (Powell 1818).

Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder affecting around 11% of the global population (Canavan et al. 2014). Currently, IBS is defined as a *disorder of gut-brain interaction* rather than a functional disorder (Drossman and Hasler 2016). In Finland, its prevalence is in a range 5.1-16.2% depending on the diagnostic criteria in use (Hillilä and Färkkilä. 2004). IBS is the largest diagnostic group seen in GI practices (Drossman et al. 2002). The health related quality of life of patients with IBS is impaired to a degree comparable to that of other chronic disorders such as depression, diabetes and inflammatory bowel disease (El-Serag et al. 2002). Furthermore, IBS related costs to the health care system are significant and absence from work is not uncommon because of IBS (Nellesen et al. 2014).

Some 40 years ago, IBS was thought to be primarily either a psychological or gut motility condition. However, it is nowadays acknowledged that IBS is likely characterised by multiple factors which include a lower than normal pain threshold in the gut and in the central nervous system (visceral sensitivity), disturbances in the gut microbiota (dysbiosis) and an altered activation of the gut immune system. The pathogenesis of IBS, similar to its clinical manifestation, is heterogeneous (Chey et al. 2015). It is likely that there are different pathophysiologies behind similar clinical symptoms (Chey et al. 2015). The clinical severity of IBS varies remarkably with some patients suffering agonies whereas others consider their symptoms as rather mild and bearable even although they may fulfil the diagnostic criteria (Drossmann et al. 2011).

The typical symptoms in IBS include abdominal pain or discomfort, bloating, flatulence, diarrhoea and/or constipation, abdominal rumbling and cramping. Many other GI-symptoms and extra-gastrointestinal symptoms such as tiredness may also occur. The current official diagnostic criteria have been set and named by the not-for-profit Rome foundation; the Rome IV criteria are the currently valid ones (Palsson et al. 2016).

The therapeutic modalities in IBS include diet, dietary supplements, drugs, physical exercise and psychological interventions such as hypnotherapy and behavioural therapy. The increased understanding of FODMAPs (Fermentable Oligo-, Di-, Monosaccharides And Polyols) has led to therapeutic applications; a low FODMAP diet has become a common treatment of IBS at least in the Western countries (Staudacher and Whelan 2017).

Grains, such as barley, buckwheat, oats, rice, rye, sorghum and wheat, are staple foods and an integral part of the daily diet all around the world. On the other hand, grains can also be viewed as an example of a food group with potentially symptom-triggering substances for individuals with IBS. The FODMAPs, gluten and possibly amylase trypsin inhibitors (ATIs) present in wheat, barley and rye seem to be one of the major reasons why these grains are often considered as major triggers of the symptoms encountered in IBS (De Giorgio et al. 2016). However, the role of the relative and comparative importance of these substances in symptom aggravation is uncertain, nor is it known which other factors may contribute to the poor tolerance of gluten-containing grains in IBS.

So-called non-coeliac gluten sensitivity (NCGS) belongs to the spectrum of functional gastrointestinal disorders and it is claimed to be caused solely by grains (De Giorgio et al. 2016). It is a new clinical entity proposed by an internationally eminent consensus group (Sapone et al. 2012). This group have postulated that NCGS is a totally new clinical entity characterised by GI symptoms triggered by gluten-containing grains in the absence of coeliac disease, wheat allergy or gluten ataxia. This consensus statement has bot been unequivocally accepted, both the existence and the role of NCGS, or non-coeliac wheat sensitivity (NCWS), are still controversial and questioned (De Giorgio et al. 2016).

The main purpose of this thesis was to examine if a reduction in the levels of FODMAPs and ATIs in grain products would improve their tolerability in subjects with IBS and NCGS. The specific aims were to study the effects of biotechnologically modified grain products on symptoms, colonic fermentation and gut microbiota, and immune activation in individuals with IBS and NCGS. The grain products used in the studies were industrially modified in order to reduce their content of FODMAPs or amylase-trypsin inhibitors. The products were prepared exclusively as breads.

2 REVIEW OF THE LITERATURE

2.1 Definition, severity, prevalence and prognosis of irritable bowel syndrome

IBS is traditionally described as a functional gastrointestinal disorder. The term "functional" refers to the absence of any organic disease causing the symptoms. The current diagnostic criteria for investigational and clinical purposes were set by the Rome Foundation. The recent version of diagnostic criteria is called the Rome IV criteria (Schmulsson and Drossman 2017). According to Rome IV criteria, IBS is defined as *a gut-brain disorder* that fulfils the following criteria:

- Recurrent abdominal pain on average at least once a week in the last 3 months associated with 2 or more of the following
    - Abdominal pain is associated with defecation and/or
    - Abdominal pain is associated with change in frequency of stool and/or
    - Abdominal pain is associated with the form (appearance) of stool

At the time of initiation of the studies included in this thesis, Rome III criteria were the latest available criteria for IBS. Rome III criteria differ mostly from Rome IV by having also "abdominal discomfort" as a diagnostic criterion in addition to pain. Other differences, perhaps of minor importance, between Rome III and IV also exist in the terms related to defecation, stool form and frequency (Samuelsson and Drossman 2017).

There has been a longstanding debate centred around the term "functional" as it is non-specific and potentially stigmatising (Schmulsson and Drossman 2017). Therefore, IBS and other functional gastrointestinal disorders were recently re-defined by the Rome IV consensus group as *disorders of gut-brain interaction* (DGBI); these are "a group of disorders classified by GI symptoms related to any combination of motility disturbances, visceral hypersensitivity, altered mucosal and immune function, gut microbiota, and/or central nervous system processing". Consequently, the Rome IV group seriously attempted to eliminate the term "functional" from their thinking and from clinical terminology regarding IBS. However, the group acknowledged the challenge in this attempt "The Rome Foundation understands that it will take time until the term functional is completely eliminated from the health care language, and in some clinical disorders the term functional was retained to distinguish them from other similar disorders (for example Functional Diarrhea) until a more appropriate term can be used."

In addition to abdominal pain or discomfort, the typical symptoms experienced by people with IBS include abdominal bloating/distension, cramps, flatulence, dyspepsia, incomplete feeling of evacuation, constipation, or diarrhoea (Wiklund 2003, Chey et al. 2015). These symptoms are typically evaluated in clinical trials and are a part of a validated instrument, i.e. GSRS (gastrointestinal symptom rating scale), intended for measuring symptoms in

IBS (Wiklund et al. 2003) albeit they are not part of the Rome diagnostic criteria. Fatigue and other non-gastrointestinal symptoms are common non-GI symptoms in IBS (Han and Yang 2016). Some other rather common non-GI symptoms or comorbidities include sleeping difficulties, palpitations, headaches, dizziness, joint and chest pain, anxiety, depression, shortness of breath and intercourse related pain (Polster et al. 2017).

The severity of IBS can be evaluated by completing a questionnaire called the "symptom severity score", i.e. IBS-SSS developed by Francis et al. (1997). This is one of the few validated instruments available for measuring IBS symptoms in clinical studies. IBS-SSS consists of five different questions; of these, the first is about the severity and the second about duration of pain, the third in concerned with bloating, the fourth assesses the overall satisfaction with bowel function with the fifth question inquiring about the overall disturbance of life (global well-being) due to bowel symptoms. Each question can receive a value 0-100, and thus the range of the total IBS-SSS score is 0-500. People whose score is under 75 are classified as healthy, 75-174 is classified as mild IBS, 175-299 as moderate and 300-500 as severe IBS (Francis et al. 1997). The functional bowel disorder severity index (FBDSI) is another tool used in the evaluation of symptom severity; this concentrates solely on abdominal pain and on the annual number of visit to doctors (Drossman et al. 1995). The FBDSI score can range from 0 to 360 but it has been rarely used in clinical studies.

The clinical severity of IBS varies remarkably; some patients suffer severely whereas some consider their symptoms as rather mild and bearable even although they fulfil the diagnostic criteria of IBS. Furthermore, the chronic symptoms of IBS follow a fluctuating course. As there was no consensus on what is severe IBS and how it should be defined, the Rome Foundation Working Team Committee produced a consensus report on the classification and definition on the severity of IBS (Drossman et al. 2011). They adopted the IBS-SSS score as a crude instrument for assessing severity (see above) but underline in the report that severity of IBS should not be understood simply as the intensity of pain or other symptoms. Rather, it is "a biopsychosocial composite of patient reported gastrointestinal and extra-intestinal symptoms, degree of disability, and illness related perceptions and behaviors". According to the report, severity can be understood as a continuum and can also be subcategorized into clinically meaningful subgroups as mild (~ 40%), moderate (~ 35%), and severe (~ 25%); the prevalence of each category and their distinguishing features will require further study. Table 1 depicts the typical features encountered in the different subgroups. A study of 172 IBS patients from Sweden suggested that 12% of IBS patients fulfilling Rome 3 criteria had mild IBS, 37% moderate and 52% severe; it is noteworthy that patients were classified by GSRS, not by IBS-SSS (Polster et al. 2017). A visual analog scale (VAS) 100 mm in length is often also used for symptom assessment but this has not been comprehensively validated; a recent review paper described both the weaknesses and strengths of these symptom assessments when conducting IBS related dietary studies (Whelan et al. 2018).

The prevalence of IBS varies according to diagnostic criteria in use. According to a systematic review, on average the global prevalence of IBS is 11% (Lovell and Ford 2012). The prevalence of IBS in Finland lies between 5-16% according Hillilä and Färkkilä (2004).

It is noteworthy that prevalence of IBS seems to decrease as a function of time because the diagnostic criteria have become more stringent, i.e. the Manning criteria from the 1980s were rather liberal with the more recent Rome criteria being more restrictive (Hillilä and Färkkilä 2004, Lovell and Ford 2012). Rome IV criteria halved the prevalence of IBS in a Chinese population when compared to the situation when Rome III was utilized (Bai et al. 2017).

Table 1. Proposed clinical profile for patient-rated severity of IBS[a] according to Drossman et al. 2011. Produced with permission from the publisher (Springer Nature, copyright clearance center)

Clinical feature	**Mild**	**Moderate**	**Severe**
Estimated prevalence	40%	35%	25%
IBS-SSS, total score	75-175	175-300	300
FBDSI	<36	36-109	>110
Physiological factors	Primarily bowel function	Bowel dysfunction and CNS pain dysregulation	Primarily CNS pain dysregulation
Psychosocial difficulties	None or mild Psychological distress	Moderate Psychological distress	Severe. High psychological distress, catastrophising, abuse history
Gender	Men=Women	Women>Men	Women>>Men
Age	Older>younger	Older=younger	Younger>older
Abdominal pain	Mild/intermittent	Moderate, frequent	Severe/very frequent or constant
Other symptoms	Low (1-3)	Medium (4-6)	High (≥7)
Health-related quality of life	Good	Fair	Poor
Health-care utilisation	0-1/year	2-4/Year	≥5/Year
Activity restriction	Occasional (0-15 days/year)	More often (15-50 days/year)	Frequent/constant (>50 days/year)
Work disability	<5%	6-10%	≥11%

CNS, central nervous system; FBDSI, Functional Bowel Disorders Severity Index; IBS, irritable bowel syndrome; IBS-SSS, IBS Severity Scoring System. [a] This is based on existing data on the severity in IBS and needs to be further tested and validated (Drossman et al. 2011).

According to a systematic review, IBS is more common among females (RR 1.67) and less common in people over 50 years (RR 0.75) (Lovell and Ford 2012). The prevalence seems to be roughly at the same level (6-10%) in the Middle East/Africa, Australasia, Europe and

North America but substantially higher in Latin America (17.5%). The authors of the systematic review speculated that inaccurate translation of the survey questionnaire and cultural differences in reporting and interpreting symptoms may explain, at least partly, the higher prevalence of IBS in Latin America.

Patients with IBS report their symptoms as occurring in exacerbation episodes (Weinland et al. 2011). Because of this fluctuation in the symptoms, or alternatively because there may be true healing of IBS, 52 % of American IBS patients initially diagnosed with IBS do not meet IBS criteria in a repeated examination at the 2-year follow-up (Williams et al. 2006). In a Danish cohort, 8% of the population had "fluctuating IBS" and 2% "persistent IBS" (Heinsveig Poulsen et al. 2015). Persistent IBS was defined as meeting Rome III criteria both at the 0 and 5 years' examination and fluctuating IBS as meeting criteria at either time point only once. These data clearly underline the fluctuating nature of symptoms. Furthermore, it is unsure if IBS is "incurable". It is not known if some patients stop experiencing symptoms after a certain point of time, or if the re-appearance of IBS symptoms is simply a matter of having a long enough follow-up time.

A possible model for finding an answer to this question is post-infectious IBS (PI-IBS). After viral, protozoal or bacterial gastroenteritis, a certain degree of the population, around 10% according to a recent systematic review, develop IBS that persists for years; this is called post-infectious IBS, PI-IBS (Klem et al. 2017). Some data included in the previous systematic review seem to indicate that the symptoms of IBS may decline during the course of time, albeit the data is somewhat inconsistent. A Korean cohort study following Shigella-infected IBS patients for ten years found that the odds ratio for IBS at ten years after the parasite-induced gastroenteritis was similar with the general population whereas at three and five years, the corresponding odds ratios were 3.91 and 1.88. These data together with the data of Heinsveig Poulsen et al. (2015) suggest that IBS may heal by itself, at least in a minority of patients.

2.2 Pathogenesis of irritable bowel syndrome

It is vital to understand the key players and their role in pathogenesis, otherwise it is challenging to appreciate why certain IBS treatments may or may not work. As stated in chapter 2.1., irritable bowel syndrome should not be defined any longer as a functional gastrointestinal disorder but as a *disorder of gut-brain interaction* (Schmulsson and Drossman 2017).

IBS has been considered as a disorder without any underlying pathological explanation for the symptoms that patients experience, but this concept is probably outdated (Holtmann et al. 2016), as the following text will demonstrate. IBS has been divided into subgroups according to the predominant stool pattern because this categorisation defines treatment. According to the Rome IV criteria, the subtypes of IBS are diarrhoea predominant IBS (IBS-D), constipation predominant IBS (IBS-C), mixed type of IBS where diarrhoea and constipation alternate (IBS-M) and finally unclassified IBS (IBS-U) in which the bowel habits cannot be accurately categorized into any of the above groups (Lacy et al. 2016).

Holtmann et al. (2016) propose that there are, in fact, several different disease mechanisms underlying these subtypes and they argue:

> *"Traditionally, irritable bowel syndrome has been considered to be a disorder with no known underlying structural or biochemical explanation, but this concept is likely to be outdated. In this review we challenge the widely accepted view that irritable bowel syndrome is an unexplained brain–gut disorder. There is epidemiological evidence that, in a major subset of patients, gastrointestinal symptoms arise first and only later do incident mood disorders occur. Additionally, possible mechanisms for gut–brain dysfunction have been identified, suggesting primary gut disturbances might be the underlying cause in a subgroup."*

The Rome Foundation, the diagnostic group for bowel disorders of Rome IV consensus has arrived at similar conclusions based on the recent studies in IBS.

> *"IBS is a multifactorial disorder with a complex pathophysiology. Factors that increase the risk of developing IBS include genetic, environmental, and psychosocial factors. Factors that trigger the onset or exacerbation of IBS symptoms include a prior gastroenteritis, food intolerances, chronic stress, diverticulitis, and surgery. The resulting pathophysiologic mechanisms are variable and patient independent, and include altered GI motility, visceral hyperalgesia, increased intestinal permeability, immune activation, altered microbiota, and disturbances in brain–gut function"* (Lacy et al. 2016)

2.2.1 Visceral sensitivity

The biological function of the pain sensation is to detect potentially harmful stimuli. However, tissue injury may not be necessary for the induction of abdominal/visceral pain, as it is for pain from skin, muscles, connective tissues or bones. Under normal conditions, the gastrointestinal tract is not a source of conscious sensory experiences apart from registration of physiological sensations such as fullness, satiety, hunger and urgency; we do not feel food moving in our gut. The gut typically fails to feel cutting, crushing or burning, but in humans, a distension of the stomach, small intestine, large bowel and rectum typically evokes sensations including pain. Unpleasant sensations are generally felt acutely or perceived as painful when stimuli exceed the normal physiological range or when submucosal nerve endings are sensitized as a consequence of tissue injury, such as in inflammatory bowel disease (IBD), gastroenteritis or bowel cancer. (Keszthelyi et al. 2012)

Visceral sensitivity, also named as visceral hyperalgesia or hypersensitivity, has long been acknowledged as a hallmark of IBS (Camillieri et al. 2001). It is a situation when a person senses gastrointestinal symptoms and especially pain at lower intraluminal pressure than healthy controls. It still has a central role in IBS, even if only 30-40% of IBS cases experience visceral sensitivity (Keszthelyi et al. 2012, Holtmann et al. 2016). Visceral

sensitivity is understood as having two distinctive domains: 1) sensitisation of afferent nerve endings at level of gut, i.e. in either submucosal or myesentric plexus of enteric nervous system (ENS) and 2) sensitised/altered handling of pain either at the cortical or spinal levels of the central nervous system (CNS). Figure 1 illustrates two focal points of visceral sensitivity (according to Keszthelyi et al. 2012).

Recent prospective cohort studies have suggested that the origin of visceral sensitivity seems to be at the level of the submucosal and myentric plexus in every second case and at the level of the CNS in the others (Koloski et al. 2012, Koloski et al. 2016). However, more studies are needed to confirm these findings.

To summarize, many, but not all patients, with IBS demonstrate disturbed handling of pain signals either at the peripheral level (afferent sensitisation in submucosal or myenteric plexus) or at the level of CNS; in the CNS, the disturbance can be detected at either the cortical or spinal level, or both.

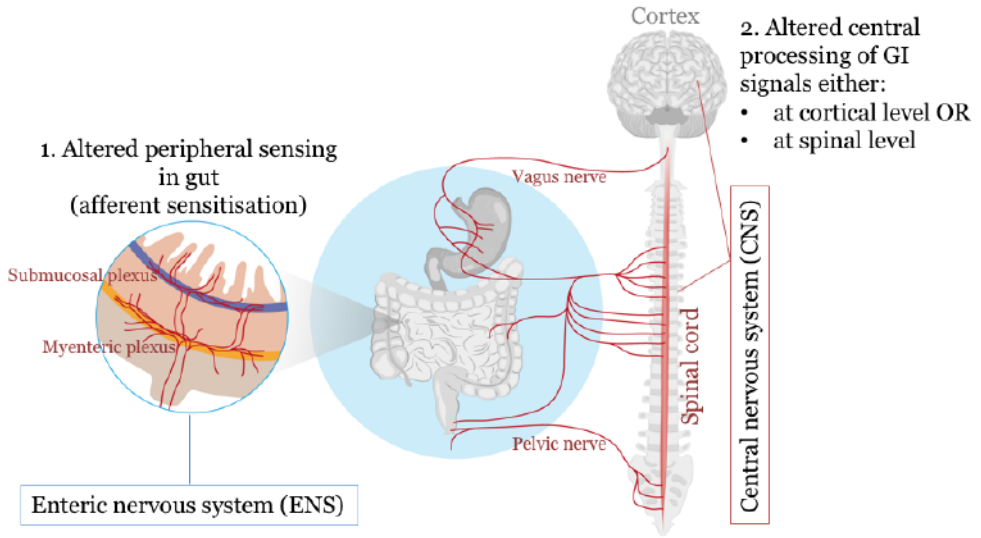


Figure 1. Schematic presentation of two focal points of disturbed pain handling in visceral sensitivity/IBS. Image drawn by Mats Vuorenjuuri for the purpose of this thesis.

2.2.2 Gut microbiota

Emerging evidence suggests that the composition of the gut microbiota is associated with many diseases such as type 2 diabetes, cardiovascular disease, cancer, allergy and neuropsychiatric diseases (Nagpal et al. 2014). It is perhaps less surprising that an altered gut microbiota has also been linked with GI disorders such as irritable bowel syndrome.

The gut microbiota comprises more than 400 species (Bennett et al. 2015) and the cell count of microbiota is estimated to be approximately the same as the total count of human cells (Sender et al. 2015). Baquero and Nombela 2012 have called it “the neglected organ”. Commensal, i.e. naturally occurring, bacteria in gut are viewed as necessary for healthy digestion, because some gut bacteria produce enzymes, such as lactase, vitamins such as vitamins K/B and metabolites such as short-chain fatty acids or branched-chain amino acids which exert either local or systemic effects on health.

The gut microbiota is also crucial for the normal development of the gut immune system (Öhman and Simren 2015). The immune system must tolerate the presence of these commensal bacteria. Furthermore, the gut immune system must recognise pathogenic bacteria from their commensal counterparts and be able to mount an attack against these pathogens in order to re-establish balance and health. The gut microbiota seems to also play a role in food allergy development; an altered gut microbiota composition early in life is an emerging factor affecting the development of food allergy (Nowak-Wegrzyn et al. 2016, Aitero et al. 2017).

Generally, the intestinal microbiota composition of healthy individuals is relatively stable after infancy (after 2-3 years of life) whereas there is extensive variability between individuals (Rajilić-Stojanović et al. 2014). However, several factors, such as antibiotic treatment, diet, stress, the host immune system and the pH of the gut milieu can affect the gut microbiota to a certain degree (Bennett et al. 2015, Maloney et al. 2016). After antibiotic treatment or a special dietary period, the microbiota tend to become restored back to their original status.

Over the past decade, the disturbances in gut microbiota in IBS have attracted increasing attention. The gut microbiota may contribute to IBS symptoms by altering gut neuromotorsensory function, barrier function or the brain-gut axis (Simrén et al. 2013). Most, if not all studies, that compare microbial profiles in IBS patients and healthy controls have been associative and cannot distinguish cause from consequence. The most convincing evidence for a causative role of microbiota in IBS is the existence of PI-IBS (Simrén et al. 2013). As stated previously, around 10% of affected people develop IBS after gastroenteritis (Klem et al. 2017). The beneficial effects of probiotics and the antibiotic, rifaximin, are other direct evidence confirming the role of microbiota in IBS; see a more detailed discussion in the following chapters “Probiotics” and “Drugs”.

Furthermore, there is growing evidence suggesting that at least subgroups of IBS patients have an altered gut microbiota composition, an abnormal site of its residence (small intestinal bacterial overgrowth, SIBO) or instability in their gut microbiota. There are several review papers and doctoral theses devoted to this theme (Kassinen 2007, Salonen and Vos 2010, Simrén et al. 2013, Jalanka 2014, Collins 2014, Rajilić-Stojanović et al. 2014, Maloney et al. 2016, Staudacher and Whelan 2016). It is unclear how alterations in the microbiota then cause symptoms or initiate the processes that lead to IBS; toxic bacterial metabolites may play a role (Campbell et al. 2010) but there may well be other factors.

The typical bacterial disturbances encountered in IBS seem to include a reduced abundance of many bacteria with potential anti-inflammatory properties such as bifidobacteria, lactobacilli and Faecalibacterium, as well as some other bacterial changes. A systematic review of 13 cohorts involving 360 IBS patients and 268 controls suggested that the abundance of lactobacilli, bifidobacteria and *Faecalibacterium prausnitzii* would be reduced in IBS (Liu et al. 2017). A sub-group analysis demonstrated that IBS-C patients had a similar abundance of lactobacilli and bifidobacteria as controls while subjects with IBS-D had the most prominent loss of these beneficial bacteria groups. On the other hand, differences in gut microbiota can be detected between IBS-D and IBS-C subtypes; an abundance of Streptococcus and Ruminococcus, and furthermore individuals with IBS-D had a more severe reduction of lactobacilli and bifidobacteria than their counterparts with IBS-M or IBS-C. *Methaninobrevibacter smithii* is a key bacterium producing methane in gut and people with IBS-C seem to have a higher abundance of this species than healthy controls or patients with IBS-D (Triantafyllou et al. 2014, Goshal et al. 2016). Furthermore, the relative abundance of *Streptococcus, Ruminococcus Roseburia, Blautia, Doria, Enterobacter* and *Enterobacteriacaea* may be increased in IBS. The biodiversity of microbiota may also be reduced in IBS (Rajilić-Stojanović et al. 2014) albeit some studies have detected no difference in microbial diversity (Dlugosz et al. 2015a). Despite extensive research efforts, it remains unclear and debatable exactly how and the extent to which these changes contribute to the symptoms or are involved in the natural course of IBS.

It is not only the abundance of certain bacterial groups that can be distorted in IBS. Finnish researchers first showed that the microbiota of subjects with IBS seems to be unstable when compared to healthy subjects (Mättö et al. 2005). They measured the composition of microbiota three times over six months (at 0, 3 and 6 months) and noted that there were more and larger changes in the microbiota of the patients with IBS when compared to healthy controls. Durbán et al. (2013) also reported instability of the microbiota in IBS-D. However, it is unclear if the instability is driven by sporadic or periodic external stimuli, i.e. dietary changes, or is it a true characteristic feature of the microbiota of IBS patients even in stable life conditions (Durbán et al. 2013). Some data imply that stress episodes might explain, at least partly, the fluctuating nature of microbiota (Mayer et al. 2014). Other factors that might be behind the instability of microbiota include periodic elimination diets in an attempt alleviate the worsening symptoms, the common use of proton pump inhibitors or antibiotics as treatment trials against excessive colonic fermentation/bloating because each of these are thought to have repercussions on the microbiota (Simrén et al. 2013).

The large bowel, the colon, harbors most of the gastrointestinal bacteria. However, IBS can be sometimes characterised by SIBO (Simrén et al. 2013). SIBO is a condition where the small intestine harbors more microbes than normally. Because microbes are able to ferment any unabsorbed carbohydrates, SIBO can cause excessive fermentation processes in small intestine, and thereby cause bloating, diarrhoea and flatulence, before the remains of the food enter the colon.

However, the diagnosis of SIBO is a challenge. The gold-standard method is the taking of an aspiration sample from jejenum but there are no universally agreed diagnostic values for aspirates (Simrén et al. 2013). The procedure involved in taking an aspirate sample from jejenum is burdensome both to the patient and physician because it involves the gastroscopy procedure. The lactulose hydrogen breath test or glucose hydrogen breath test are more often used in clinical studies but these tests are confounded by several factors; and their clinical value remains unclear (Simrén et al. 2013). Nonetheless, despite the lack of universally agreed diagnostic criteria and standardized methods, it is estimated that 4%-78% of patients with IBS and from 1% up to 40% of healthy controls have SIBO, and many experts believe that SIBO has some relevance to IBS (Ghoshal et al. 2017).

In summary, it can be stated that individuals with IBS display several disturbances in gut microbiota; the abundance of several bacterial groups is altered, microbiota can be labile and microbes can be harboured in the small intestine in abnormal amounts (SIBO). The exact role of the gut microbiota is still being elucidated; however, the microbiota appears to be one of several important factors that contributes to the etiology and pathophysiology of IBS. The most convincing piece of evidence that ties the gut microbiota into IBS is the very existence of post-infectious IBS.

2.2.3 Increased intestinal permeability

The surface of both the small and large bowels is composed of simple columnar cells. These cells of the gut lining are called enterocytes. There are so-called tight junctions between enterocytes that control the intracellular space. Tight junctions allow absorption of nutrients, but on the other hand, form an epithelial barrier against pathogens and harmful substances (Ulluwishewa et al. 2011).

The regulation of tight junctions seems to be disturbed at least in subsets of IBS-D patients (Piche 2014). In subjects with IBS-D and PI-IBS, the tight junctions may become loosened leading to increased intestinal permeability, this is colloquially termed as “leaky gut” (Camilleri et al. 2012, Quigley 2016). Food and microbial antigens, such as toxins, proteins and peptides may thereby stimulate the mucosal immune system and cross the intestinal border and more easily gain access to the circulation. Furthermore, water can pass from the body into the lumen of the gut thus promoting the development of diarrhoea. Both increased penetration of harmful substances and the flux of water into lumen can cause the GI and non-GI symptoms related to IBS (Matricon et al. 2012).

The translocation of a probe molecule across the intestinal epithelium is the basic concept used when assessing intestinal permability. There are several commonly used probe molecules inpermeability tests in IBS e.g. lactulose, mannitol and polyethylene glycols; indirect measures of permeability also include molecules such as zonulin, occludin or claudin-1 (Bertiaux-Vandaële N et al. 2011, Kerckhoffs et al. 2010, Rao et al. 2010). None of these tests has become the established gold standard method in IBS.

Zhou et al. (2018) fed rats a diet high in FODMAPs and observed an increase in faecal Gram-negative bacteria and serum lipopolysaccharide (LPS) levels, together with barrier dysfunction, increased intestinal inflammation and visceral hypersensitivity. In addition, a low FODMAP diet was found to reduce LPS levels and improve symptoms in human subjects with IBS, albeit the number of patients in the trial was low. These data suggest that a so-called high FODMAP diet (normal diet) can increase the systemic exposure to LPS in IBS due to increased permeability of the gut lining.

One way in which hyperpermeability may take place in IBS-D is via altered microRNA (miRNA) expression profile (Wouters 2017). MiRNAs are particularly relevant for intestinal disorders as recent research has described their impact in the regulation of immune and inflammatory responses. Alterations in miRNAs lead to reduced glutamine synthesis since glutamine plays a crucial role in maintaining intestinal barrier function and its depletion leads to villus atrophy, decreased expression of the proteins needed for tight junctions and increased intestinal permeability (Wouters 2017). In an innovative confocal microscopy study, Turcotte et al. (2014) found that IBS patients had significantly more epithelial gaps in their small intestine as compared with healthy controls. The median gap density of IBS patients was 32 (range 17-42) gaps/1000 cells versus 6 (range 0-13) gaps/1000 cells for healthy controls (P =0.001). Thus, the authors concluded "Increased epithelial cell extrusion may be a cause of altered intestinal permeability observed in IBS".

It is challenging to determine the actual role of intestinal hyperpermability because intestinal permeability is interrelated to gut microbiota, stress, diet and many other factors also contributing to the etiology of IBS (Simrén et al. 2013). However, experts agree that intestinal permeability is probably one of the mechanisms behind the symptoms encountered in IBS.

2.2.4 Immune activation and intestinal inflammation

IBS is not an overt inflammatory disease in contrast to Crohn's disease or ulcerative colitis, which are commonly called inflammatory bowel diseases (IBD). There are no detectable inflammatory lesions on the gut wall, neither is there any increase in conventional markers of inflammation such as serum CRP, erythrocyte sedimentation rate or fecal calprotectin in IBS (Spiller and Major 2016). However, more subtle inflammatory changes, i.e. low-grade inflammation or immune activation at the level of the mucosa can be observed, at least in the subset of people with IBS. One of the initial findings on low-grade inflammation and increased permeability was made by Finnish researchers from the University of Helsinki; in their innovative study, Kajander et al. (2009) demonstrated that the mucosa of IBS patients could be characterised by a distinct pro-inflammatory and lipotoxic metabolic profile. In particular, there was an increase in the levels of several lipids such as lysophospholipids and ceramides.

With regards to elevated immune activation in IBS, the general consensus and the most convincing body of evidence highlight the role of mast cells (Ford and Talley 2011, Wouters et al. 2016). Elevated mast cell activation and/or an increased abundance of mast cells over other immunocytes are common findings in the mucosa of patients with IBS (Simrén et al.

2013). Mast cells are mainly located in the lamina propria of mucous membrane of the gut lining; these cells are best known as key producers of histamine. Apart from histamine, mast cells produce tryptase and other proteases, serotonin, interleukins, leukotrienes, prostaglandins and TNF-α and some other pro-inflammatory substances (Zhang et al. 2016). It is rather well established that mast cell activation can generate epithelial and neuromuscular dysfunction and promote visceral hypersensitivity and altered motility patterns in IBS, postoperative ileus, food allergy and inflammatory bowel disease (Wouters et al. 2016). Ostertag et al. (2015) reported that mucosal biopsies of IBS patients seemed to mount a weakened activation to a mixture of histamine, serotonin, tryptase, and TNF-α when compared to healthy controls; the researchers argued that this was due to a desensitization towards the mediators constantly released by mucosal cells of IBS patients.

One sophisticated study in humans showed that supernatants from colonic mucosa of IBS patients, but not from healthy controls, activated human enteric neurons *in vitro* whereas supernatants (Buhner et al. 2009). In addition, the IBS supernatant-evoked excitation was mediated by proteases such as tryptase, histamine, and serotonin, with the proteases appearing to play the greatest role. These results of Buhner et al. (2009) further emphasize that altered signaling from the mucosa to the ENS may be a relevant factor in the pathophysiology of IBS. These researchers speculated that mast cells and enterochromaffin cells in the supernatant samples were likely to be the sources responsible for the supernatant-mediated excitation. Indeed, a systematic review concluded that there is some evidence that enterochromaffin cell density is elevated in IBS (Martin-Viñas and Quickley et al. 2016).

Many drugs that affect the cascades occurring in mast cells are also being developed and tested (Zhang et al. 2016). In fact, ebastine, an oral anti-histamine i.e a histamine receptor blocker, reduced visceral sensitivity and consequently abdominal pain in IBS in a randomised 3-month trial (Wouters et al. 2016) and a low-FODMAP diet has dramatically reduced the levels of histamine-metabolites in urine samples of IBS patients (McIntosh et al. 2017).

In addition to increased activation and a greater abundance of mast cells and enterochromaffin cells, several other subtle inflammatory processes have been described in IBS. The numbers of T lymphocytes, B lymphocytes, and mucosal cytokine production appear to be altered among cases with IBS, especially among those with IBS-D (Ford and Talley 2011). Two case-control studies have reported that patients with IBS exhibit higher levels of high-sensitive CRP when compared to healthy controls (Hod et al. 2011, 2016). According to a systematic review, TNF-α levels were higher in female patients with IBS than in healthy controls although no difference could be found in males (Bashashati et al. 2014). The same review also described a significantly lower serum/plasma IL-10 levels in male patients with IBS vs male controls.

Furthermore, one meta-analysis has shown that there are associations between cytokine gene polymorphisms and IBS (Bashashati et al. 2012). Carriers of the high producer anti-inflammatory cytokine IL-10 gene polymorphism were less likely to have IBS. Polymorphisms in the tumor necrosis factor (−308 G/A) gene have been associated with

IBS in Asian but not in Caucasian populations (Bashashati et al. 2012). One study also proposed that children with IBS tended to produce lower amounts of the anti-inflammatory cytokine IL-10 at baseline and after LPS stimulation, implying that defects in immune modulation may contribute to IBS in children (Hua et al. 2011). Another case-control study found that the levels or lipopolysaccharide (LPS) and antibodies to flagellin were higher in IBS-D than in healthy controls (Dlugosz et al. 2015b). Finally, a recent study reported increased expression of Toll-like receptors 4, 5, and 9 in small bowel mucosa from subjects with IBS (Dlugosz et al. 2017). These studies on systemic markers of inflammation suggest that IBS patients may have an activated immune system when compared to healthy controls.

Microscopic colitis is an inflammatory disorder affecting the colon characterised by long-lasting unresolved diarrhoea. Inflammation cannot be verified in regular colonoscopy, instead histological analysis is needed for a diagnosis, thus a biopsy specimen from colon is required for diagnosis. It is not known how often microscopic colitis is the actual cause of a condition interpreted as IBS-D but recent studies suggest that at least 4% of IBS-D cases might be explained by microscopic colitis (Ozdil et al. 2011, Stoicescu et al. 2012, Hilpüsch et al. 2017). It is impossible to differentiate IBS-D and microscopic colitis purely on the basis of the patient's clinical presentation, but from the clinical point of view, the differentiation is crucial; microscopic colitis usually responds to anti-inflammatory drugs or corticosteroids (Gentile and Yen 2017) whereas IBS-D does not. It is not known if IBS-D can convert into microscopic colitis, or the other way round. What does seem certain is that microscopic colitis might be mistaken as IBS-D.

Taken together, an abnormally activated mucosal immune system/low-grade inflammation together with the previously mentioned disturbances in gut microbiota and intestinal permeability are all factors that now challenge the traditional view of IBS as a purely functional disorder and suggest that IBS might have an organic cause.

2.2.5 Gastrointestinal cells and hormones

The gastrointestinal tract contains at least 15 different types of endocrine cells that are spread among the epithelial cells of the mucosa. These cells, which constitute about 1% of all epithelial cells in the gastrointestinal tract, have specialized sensors in the form of microvilli that sense luminal conditions and respond to luminal stimuli (diet) by releasing hormones (El-Salhy 2015). Prominent cells in lower GI tract include enterochromaffin cells, Delta cells (D cells) and L cells (Gunawardene et al. 2011). Enterochromaffin cells secrete serotonin and histamine, D cell somatostatin and L cells neurotensin and glucagon-like peptide-1, pancreatic peptide YY, oxyntomodulin and glucagon-like peptide-2. Pinealocytes and enterochromaffin cells in GI tract produce also melatonin (Chojnacki et al. 2013).

Gastrointestinal hormones may have a role in the pathophysiology of IBS. It has been argued that the density of intestinal endocrine cells is reduced in "normal" IBS patients but increased in PI-IBS (El-Salhy et al. 2017). The reduction in the numbers of gastrointestinal endocrine cells seems to be caused by abnormal clonogenic and differentiation activities of

the intestinal stem cells (El-Salhy et al. 2017); they proposed that the abnormalities in the gastrointestinal endocrine cells explain the visceral hypersensitivity, disturbed gastrointestinal motility, and the abnormal gut secretion observed in subjects with IBS. It is of considerable interest to nutritionists and dietitians that a low-FODMAP diet seems to restore the density of endocrine cells in IBS (Mazzavi et al. 2015). However, these results will require confirmation from other research teams; the reduced density of endocrine cells has not been confirmed but neither has it been refuted.

Serotonin secreted from enterochrommaffin cells is thought to play a role in IBS according to Martin-Viñas and Quickley et al. (2016). The GI tract contains 90% of the body's serotonin stores. Conventional functions of serotonin are perceived as intrinsic reflexes (e.g. stimulation of propulsive and segmentation motility, epithelial secretion and vasodilation), and the activation of extrinsic vagal and spinal afferents that alter gastric emptying, pancreatic secretion, feelings of satiation, pain and discomfort and may mediate nausea and vomiting and furthermore serotonin may also promote inflammation (Camillieri 2012, Holtmann et al. 2016). There is evidence that there are abnormalities in serotonin metabolism in IBS. Serotonin levels seem to be higher in IBS-C and lower in IBS-D when analysed from platelet-depleted plasma (Camillieri 2012, Holtmann et al. 2016). It has also been suggested that IBS patients with diarrhoea might have reduced serotonin re-uptake, and those with IBS with constipation might have impaired release of serotonin (Camillieri 2012, Holtmann et al. 2016). Both the serotonin receptor 4 agonist (tegaserod) and the receptor-type 3 antagonist (alosetron) have been used to treat IBS-C and IBS-D (Camillieri 2012, Holtmann et al. 2016).

The levels of melatonin in the gastrointestinal tract exceed by 10-100 times the blood concentrations. On average, there is 400 times more melatonin in the gut than in the pineal gland. Melatonin is produced from its precursor serotonin (Esteban-Zubero et al 2017). Melatonin possesses anti-inflammatory, analgesic, sleep promoting and GI motility regulating properties. Treatment with melatonin 3 mg/day at bedtime has reduced abdominal pain and visceral sensitivity in IBS (Song et al. 2005). Melatonin supplementation has been proposed to improve abdominal pain, but more studies will be needed to confirm these findings and to understand the exact mechanisms of action (Siah et al. 2014).

Goblet cells are also located on the mucosa of gut lining and their function is to produce a gel-forming mucus which protects the enterocytes from pathogens as well as the physiological irritation caused by dietary components. Research efforts in IBS have been limited but dysregulation of mucus metabolism has been postulated as one key feature in IBS (Qin 2011). IL-10 is an anti-inflammatory cytokine that stimulates the formation of mucus via Goblet cells (Birchenough et al. 2015). IL-10 seems to be downregulated in IBS, as previously discussed in this text related to inflammation and immune activation. IBS-D patients commonly describe their stools as mucus-like (Manning et al. 1978) which may further point to a dysregulation of Goblet cells and disturbed mucin metabolism in IBS. Indeed, increased mucus production and thinning of the mucus layer seemed to predominate in subjects with IBS in a clinical study (Dorofeyev et al. 2011).

It seems reasonable to propose that IBS patients have disturbances in the endocrine system of GI when compared to healthy subjects. Determining the relative importance of these disturbances and inter-connections to microbiota, low-grade inflammation, ENS and CNS remains a challenge.

2.2.6 Intestinal gas

Intestinal gas is mainly a product of bacterial fermentation in colon and to a lesser extent in the small bowel. As visceral sensitivity is one hallmark of IBS, one can understand that either higher production of gas or its slower disappearance may cause abdominal pain and feelings of distension.

Several gases are produced in the gut e.g. hydrogen, methane and carbon dioxide and hydrogen sulphide. There is convincing evidence that the handling of infused intestinal gas is impaired in IBS; a key feature being slow gas transit and retention of gas (Serra et al. 2001, Hernando-Harder et al. 2006, Salvioli et al 2008, Hernando-Harder et al. 2010,). In contrast, feeding with fermentable carbohydrates such as inulin or fructose did not result in differences in intraintestinal gas volumes which were assessed with magnetic resonance imaging with 5 hours' follow up (Major et al. 2017). It remains unclear why gas caused by inulin/fructose feeding does not seem to behave in the same way as jejunal infusion of gas. One speculative explanation can be that the infusions simply contain more gas than 40 grams of inulin or fructose are able to produce; an alternative explanation is that the infusion site (jejenum) is not so well equipped to handle gas as colon/rectum (Harder et al. 2003). Furthermore, in the normal postprandial state, the colon is the major source of intestinal gas rather than the small bowel (jejunum).

IBS patients may produce more intestinal gas than healthy controls at least in the fasted state (Kumar et al. 2010). Some studies have detected increased production of gas in IBS in the post-prandial state (Ong et al. 2011), as measured by hydrogen excretion, but not confirmed by others (Yao et al. 2014, Major et al. 2017).

Individuals with IBS-C seem to produce more methane than healthy controls or people with IBS-D (Chatterjee et al. 2007, Kunkel et al. 2011, Goshal et al. 2016). Although thought of as an inert gas, there is evidence that methane can act like a neuromuscular transmitter, resulting in reduced propagation of the peristaltic movement in the intestine (Triantafyllou et al. 2014). *Methaninobrevibacter smithii* is a key bacterium producing methane in the gut and people with IBS-C seem have a higher abundance of this bacterium in their gut (Goshal et al. 2016, Triantafyllou et al. 2014). The roles of nitric oxide and carbon dioxide in IBS are far from clear, no studies exist on their specific role in IBS.

It seems that persons with IBS experience a slower disappearance of intestinal gas but the location of the defect is unclear; in the evacuation process (flatus), in its excretion into breath or in the consumption of gases by gut microbes. According to a meta-analysis, breath testing was four times more often abnormal among IBS subjects than among healthy controls; this implies that there is altered handling or production of gas in IBS patients (Shah et al. 2010).

2.2.7 Impaired intestinal motility

IBS is conventionally classified according to GI motility into constipation or diarrhoea predominant or mixed IBS. During the last decade, unclassified IBS (IBS-U) has also been added because not all patients have clear motility disturbances. Nevertheless, it is estimated that more than 75% of IBS patients experience motility problems, i.e. they have either IBS-C, IBS-D, or IBS-M (Ersryd et al. 2007, Yao et al. 2012). As described earlier, colonic methanogenesis seems to be associated with constipation whereas more severe inflammation appears to be associated with IBS-D. In patients with co-existing functional dyspepsia, gastric emptying can be slowed and gastric accommodation to a food bolus can be impaired. Since these mechanisms cause the stomach to be more full, this can provoke symptoms in the upper GI tract in the presence of visceral sensitivity (Haag et al, 2004, Farré et al. 2013, Steinsvik et al. 2016).

One intriguing observation is that IBS patients seem to have intra-abdominal volume displacement. Patients with IBS-related bloating show an impaired abdomino-phrenic coordination with a paradoxical contraction of the diaphragm coupled with a relaxation of the abdominal wall, leading to an increase in abdominal circumference (Iovino et al. 2014). A functional MRI study revealed that IBS-C patients had a dilated transversal colon and IBS-D and IBS-M patients had a constricted small bowel which was speculated to be due to a decreased level of water in the small bowel in IBS-C (Lam et al. 2017).

Taken together, these data confirm that the majority of IBS patients suffer from altered motility of the GI tract but there are also IBS patients that do not have motility disturbances, i.e. they have IBS-U.

2.2.8 Short-chain fatty acids

Commensal bacteria harboured in the distal intestine consume the carbohydrates that have not been absorbed in small intestine. Dietary fibre is the most important carbohydrate with respect to this bacterial fermentative process. As a result of the fermentation, intestinal gas and short-chain fatty acids (SCFAs) are formed with butyrate, propionate and acetate being the most abundant SCFAs. Short-chain fatty acids have many regulatory functions, such as enhancement of electrolyte and water absorption in colon (Camillieri et al. 2015). The anti-inflammatory role of butyrate is of major interest since butyrate is extensively formed from dietary fibre and is the key fuel for enterocytes. A depletion of dietary fibre, i.e. a low fibre diet, results in the reduced production of butyrate and this may cause a thinning of the mucus layer in the gut and cause increased permeability (Scott et al. 2013, Stilling et al. 2016).

Little is known about the relevance of short-chain fatty acids in IBS. A Norwegian lactulose-feeding study suggested that IBS patients, possibly due their altered microbiota, might have reduced capacity to produce short-chain fatty acids from unabsorbed carbohydrate lactulose (Undseth et al. 2015). In that study, the amount of short-chain acids was measured in the blood. Previously, it had been reported that fecal microbiota

from IBS patients produced less SCFAs in an in vitro fermentation system in response to various carbohydrates and fibers (Treem et al. 1996). An intervention study showed that encapsuled butyrate reduced defecation associated pain among IBS patients when compared to healthy controls but no difference was observed in overall abdominal pain or in other symptoms (Banasiewicz et al. 2013). In contrast to these findings, the study of Tana et al. (2015) demonstrated that fecal samples of IBS patients contained more acetate and propionate than those taken from healthy controls. In a more recent study (Ringel-Kulka et al. 2016), the total SCFA level in IBS subjects was similar with that of healthy controls but it was reduced in IBS-C.

It is premature to draw any definitive conclusions on the role of SCFAs in IBS given the somewhat contradictory results in the literature.

2.2.9 Heredity

Relatives of a person with IBS are 2 to 3 times more likely to have IBS, and studies comparing homozygotic and dizygotic twins have demonstrated that IBS is two times more likely among homozygotic than among dizygotic twins if one of the siblings has IBS (Levy et al. 2001, Saito 2011). The genetic susceptibility in twin studies has varied from 1% up to 20% and heritability estimates have ranged between 0–57% in four studies (Saito 2011). As both dizygotic and homozygotic twins share the same environment but only homozygotic twins share all the genes, this implies that genes do play some role in the development of IBS. Genetic studies have thus far focused on detecting gene polymorphisms and indeed several possible gene polymorphisms have been found to associate with IBS; however, genome wide associations studies (GWAS) are generally lacking (Makker et al. 2015). Thus far, the only published GWAS study identified one locus on chromosome 7p22.1 which included the genes KDELR2 (KDEL endoplasmic reticulum protein retention receptor 2) and GRID2IP (glutamate receptor, ionotropic, delta 2 interacting protein) and showed a consistent increasing risk in the index GWAS when all cohorts were included (Ek et al. 2015).

One rather large and recent promising example is the association between polymorphism of the gene coding for sucrose-isomaltase enzyme and IBS. The presence of sucrose-isomaltase gene variants coding for disaccharidases with defective or reduced enzymatic activity increased the risk of IBS by twofold and seemed to predispose to IBS in a cohort of 1887 individuals with IBS (Henström et al. 2018). Polymorphism in the sucrose-isomaltase gene reduced the enzymatic activity by 35%. Naturally, such a reduction may cause a reduced absorption of carbohydrates and consequently elevated intestinal fermentation of carbohydrate and retention of water in the small intestine, and ultimately this could provoke the symptoms encountered in IBS.

It can be concluded that genes seem to play a role in the development of IBS but more and larger studies will be needed to understand the underpinning physiological mechanisms and specific genes that are affected. More than 15 gene polymorphisms have been found to

associate with IBS but so far, genes are not considered as the driver of the incidence of IBS (Makker et al. 2015).

2.2.10 Diverticulosis

Recent data suggests that over half of populations over 60 years have diverticulosis. The prevalence of diverticular disease is as high as 65% by 85 years of age as compared to an incidence as low as 5% in those 40 years of age or younger. (Weizman and Nguyen 2011)

A Japanese case-control study suggested that left-sided or bilateral diverticulosis was associated with IBS whereas right-sided diverticulosis was not, the odds ratios being 3.1 for left-sided and 2.6 for bilateral diverticulosis (Yamada et al. 2014). A study exploiting the Veterans Administration Medical Centre database showed that after admission to hospital for diverticulitis, i.e. symptomatic diverticulosis, there was a 4.7-fold increased risk for developing IBS over the next 6 years (Humes et al. 2012). Furthermore, in another American prospective case-control study, patients with diverticulitis were 4.7-fold more likely to be diagnosed with IBS after a 6.3 years' follow-up (Cohen et al. 2014). The authors proposed that this disorder should be called postdiverticulitis IBS. The underlying mechanisms in diverticular diseases include visceral hypersensitivity which may be postinflammatory but altered central pain processing also seems to be present (Spiller 2016). These represent the same mechanism that is commonly present IBS.

More data is needed but recent prospective studies have suggested that symptomatic diverticulosis is a predisposing factor for IBS.

2.2.11 Bile acids

A recent meta-analysis demonstrated that 28% of patients with IBS-D displayed a reduced absorption of bile acids (Slattery et al. 2015) and malabsorption of bile acids can lead to bile acid diarrhoea. The presence of primary bile acids in feces and blood has been correlated with pain in IBS (Dior et al. 2016). Microbes in the beginning of the colon deconjugate primary bile acids to secondary bile acids which are more hydrophobic, and thus more likely to cause diarrhoea, and more toxic than the primary bile acids. An increased level of bile acids in the proximal colon can cause microbial and pro-inflammatory changes in colon that perpetuate IBS (Pavlidis et al. 2015).

2.2.12 Psychosocial factors

Stressful life events, poor coping ability and abuse may predispose to IBS (Chitkara et al. 2009). Furthermore, individuals with IBS suffer more often than the general population from anxiety and depression. Acute experimental stress causes intestinal hyperpermeability and immune activation in human experiments performed either as cold

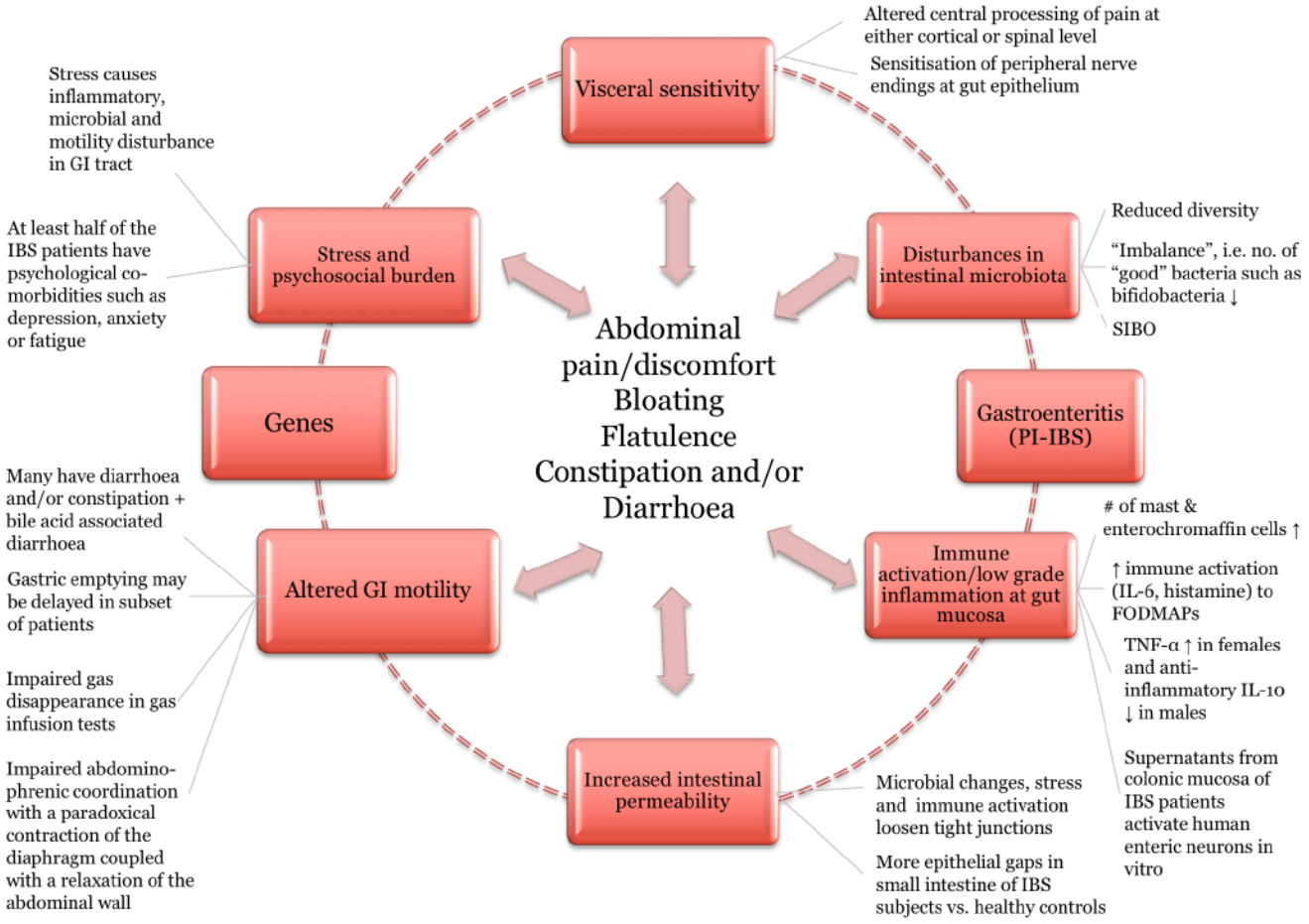
water immersion or a public speaking test (Alonso et al. 2011, Vanuytsel et la. 2014). Furthermore, experimental studies have shown that acute stressful events, such as the Trier Social Stress Test, can trigger the symptoms of IBS (Murray et al. 2004, Kennedy et al. 2014). Thus, it seems clear that brain and gut interact in generating the symptoms of IBS. It has been suggested that IBS patients with severe symptoms have more prominent psychosocial disruptions which act as both an etiological factor leading to IBS and as a co-morbidity when already suffering from IBS (Drossman et al. 2011). Maternal separation increases visceral sensitivity and therefore it has been occasionally adopted as an animal model of IBS (Moloney et al. 2012).

Stress triggers the hypothalamus-pituitary axis and the activation of the autonomic nervous system, an increase in the levels of cortisol and proinflammatory cytokines. Stress enhances the intestinal permeability by weakening the tight junctions and thus leading to increased bacterial translocation into the intestinal wall. An increased microbial load in the colonic tissue and excessive cytokine release or mast cell activation, i.e. histamine release, may then cause IBS-like symptoms. (Konteruk et al. 2011).

2.2.13 Conclusion

The IBS disease process is multifactorial and complicated, as described in the text. In the future, IBS is likely to be split into more precise and descriptive clinical sub-entities as our understanding of the disease's pathophysiology increases. Figure 2 depicts the major factors associated with IBS according to our current knowledge.

Figure 2. IBS is a multifactorial disorder which is characterised by dysfunction of gut-brain interaction.



2.3 Non-coeliac gluten/wheat sensitivity –a hypothetical clinical entity related to gluten containing grains

Non-coeliac gluten sensitivity (NCGS), also known as non-coeliac wheat sensitivity (NCWS), has attracted attention both in the scientific and popular literature (Hadjivassiliou et al. 2010, Aziz et al. 2012, Fasono et al. 2015, Time magazine April 29, 2015). The self-diagnosed prevalence of NCGS varies hugely between the studies; a systematic review of six cohorts revealed a prevalence range of 0.5%-13% (Molina-Infante et al 2015). Despite interest and publicity around gluten free diets, the biology underlying NCGS is unclear.

In 2012, an international consensus group proposed that NCGS would be a new clinical entity characterised by GI symptoms triggered by gluten-containing grains in the absence of coeliac disease, wheat allergy or gluten ataxia. (Sapone et al. 2012). At the time of the publication of this report, there was only one randomised study suggesting that NCGS really exists (Biesiekierski et al. 2011,a). Since the publication of the consensus report, several randomised studies have been published. A review of these studies was published 2016 (Volta et al. 2016).

There is no single biochemical marker of NCGS/NCWS available, the only reliable way to determine the true existence of NCGS/NCWS is to conduct high-quality blinded tests in clinical settings (DeGiorgio et al. 2016). The outcomes and features of these randomised studies in NCGS/NCWS were compiled in a systematic review and meta-analysis (Lionetti et al. 2017). Two randomised gluten sensitivity studies have been published since Lionetti's meta-analysis (Skodje et al. 2017, Dale et al. 2018), and their results are in line with the previous studies. It seems to be seldom the case that gluten per se causes an aggravation of symptoms.

In their meta-analysis of randomized studies, Lionetti et al. (2017) concluded: "the meta-analysis of the existing literature on re-challenge studies in patients diagnosed with NCGS shows that the prevalence of confirmed NCGS after re-challenge test is low". Data of these trials will be scrutinized in detail.

There has been obvious heterogeneity in the designs of NCGS/NCWS studies. The duration, method of comparison, vehicle product of gluten, nature of placebo and background diets have differed substantially from study to study. In some trials, IBS was screened at participant recruitment while in the others it was not, and consequently there was extensive heterogeneity in the participants. These methodological differences between the studies make the comparison of studies very challenging. Nonetheless, it seems that, at maximum, 14-30% of people with self-diagnosed NCGS/NCWS truly react specifically to gluten in blinded protocols (Volta et al. 2016).

One clear weakness in these studies has been the short duration of the randomised period; only one study lasted longer than 2 weeks (Biesiekierski et al. 2011, a) and another

weakness has been the selection of placebo. No one knows if whey protein, rice starch, xylose, gluten free flour or something else constitutes a gold standard placebo for studies on NCGS/NCWS. It can be argued that rice starch should be good because rice does not contain a significant amount of gluten, ATI or FODMAPs (Biesiekierski et al. 2011,b, Zevallos et al. 2017). Whey protein might also be a good placebo because it is also FODMAP, gluten and ATI free. Furthermore, according to preliminary human and experimental evidence, whey protein may improve the epithelial barrier and exert a mild anti-inflammatory effect (Benjamin et al. 2012 and Kotler et al. 2013), and whey protein alone was not able to trigger symptoms in people with non-allergic milk sensitivity (Bengtson et al. 1997).

There are also several observational interventions in NCGS/NCWS. They have inherent weaknesses but often these trials have a much longer duration than randomized studies in this particular field, thus tackling the weakness of very short intervention period typical to randomised NCGS/NCWS trials. In a 6-month non-controlled intervention, GFD decreased severity of GI symptoms; participants were not screened for IBS status at enrollment (Uhde et al. 2016). It was interesting that many markers of low-grade inflammation and intestinal permeability improved, e.g. circulating concentrations of IL-8, IL-6, FABP2 and LBP were reduced. The authors interpreted the results as follows: "These findings reveal a state of systemic immune activation in conjunction with a compromised intestinal epithelium affecting a subset of individuals who experience sensitivity to wheat in the absence of coeliac disease".

Furthermore, Sapone et al. (2011) showed after a 4-month gluten-containing diet that innate immune activation at the level of duodenal epithelium was increased among subjects with NCGS when compared to controls (patients with dyspepsia about to undergo gastroscopy). Specifically, the expression of Toll-like receptor (TLR) 2 was increased in NCGS but not in patients with coeliac disease and the expression of the T-regulatory cell marker FOXP3 was significantly reduced in gluten sensitive persons in comparison to controls and coeliac patients.

Other non-controlled studies principally support the concept of NCGS/NCWS both from the symptomatic and low-grade inflammation points of view (Volta et al. 2016). However, the inherent weakness of non-controlled trials must be taken into account; the placebo effect is always a challenge in open non-controlled studies and can have a substantial impact in disorders in which responses are being analysed on the basis of subjective ratings (Staudacher et al. 2017a). The placebo effect, the anticipated alleviation of symptoms, can skew the results towards false positive findings.

A Dutch cohort study offers an interesting perspective on NCGS (van Gils et al. 2016). In this study, individuals with self-diagnosed NCGS were asked about their sensitivity to other food items typically high in FODMAPs. It was claimed that people with NCGS are more likely to have several other food intolerances; the odds ratios for experiencing symptoms from high-FODMAP foods was found to be 3-16 higher among the individuals with NCGS when compared to controls. Abdominal discomfort related to FODMAP containing food was more often reported in NCGS individuals as compared to the control

population (73.5% vs. 21.7%). This population study suggests that individuals with self-diagnosed NCGS are very likely to be sensitive to FODMAP and may actually represent a subset of IBS.

Ultimately, a recent well-controlled 1-week randomised trial demonstrated that people with self-diagnosed NCGS do no react to gluten more often than to placebo but do react to a FODMAP, to fructan in this case (Skodje et al. 2017). In other words, unlike fructan, gluten was not able to induce symptoms more often than placebo. This study is the most convincing piece of evidence questioning the existence of pure NCGS. In line with the study of Skodje et al. (2017), an Italian randomised study, which has been published only as a congress abstract, demonstrated that individuals with NCGS react more often to FODMAPs than to gluten (Zanini et al. 2014).

Role of amylase trypsin inhibitors (ATIs) in NCGS/NCWS

Wheat, barley and rye contain FODMAPs, gluten and ATIs which all might play a role in triggering the symptoms of NCGS/NCWS. The role of gluten has been discussed previously, and a more detailed description of the role of FODMAPs will follow in chapter 2.4.1. However, there is also a need to understand role of ATIs.

Gluten is the dominating protein in wheat, rye and barley but wheat also contains other proteins such as ATIs (Zevallos et al. 2017). ATIs are defensive proteins produced by plants to combat pests. ATIs may trigger the innate immune response and induce intestinal inflammation in the host. Currently, no randomised studies have been performed with ATIs in IBS or NCGS/NCWS. However, the pre-clinical data is rather convincing; ATIs are pro-inflammatory in vivo in animals and in vitro exploiting different kind of human cell lines (Junker et al. 2012, Zevallos et al. 2017).
The report from Junker et al. (2012) demonstrated that the ATIs commonly found in wheat, barley and rye are activators of Toll-Like Receptors 4 (TLR4) and therefore they can induce an innate immune response. ATIs elicit release of proinflammatory cytokines in cells from coeliac and noncoeliac patients and in biopsies taken from coeliac patients. Mice deficient in TLR4 or TLR4 signalling were found to be protected from intestinal and systemic immune responses after an oral challenge with ATIs. Zevallos et al. (2017) found that ATIs were highly resistant to heat and digestive enzymes (intestinal proteolysis). They also concluded that gluten-containing foods contained 100-fold higher amounts of ATIs than in most gluten-free foods. Processed or baked foods retained their ATI bioactivity. The authors stated: “Most older wheat variants (such as Emmer or Einkorn) had lower bioactivity than modern wheat. ATI species CM3 and 0.19 were the most prevalent activators of TLR4 in wheat. Ingestion of ATIs induced modest intestinal myeloid cell infiltration and activation, and release of inflammatory mediators—mostly in the colon, followed by the ileum, and then in the duodenum. Dendritic cells became prominently activated in mesenteric lymph nodes. Concentrations of ATIs found in a normal daily gluten-containing diet increased low-level intestinal inflammation [in mice with experimental colitis.]”. These authors claimed that modern wheat has become enriched with ATIs because it has been bred to be highly resistant to attacks from pests.

These are interesting findings but caution is warranted in interpreting the studies. In fact, ATIs can be found in gluten preparations (Junker et al. 2012, Zevalllos et al. 2016); consequently, when gluten preparations or gluten grains are used in any randomised study; these trials test the ATI hypothesis, at least indirectly. Interestingly, the authors did not address this issue when reviewing the literature in their paper (Zevallos et al. 2017).

Furthermore, the effect of ATIs may be a matter of dose; "it is the dose that makes the poison" is the basic principle underlying toxicology. On the basis of in vivo and experimental studies, it has not been possible to determine a safe or hazardous dose for susceptible individuals. Furthermore, people have been consuming wheat, barley and rye for thousands of years, and therefore as a species, humans have been challenged by ATIs for millennia i.e. for as long as we have been consuming wheat, barley and rye. It is also crucial that these results should be confirmed by the broader research community; in scientific research, the replication of a phenomenon can verify the very existence of that phenomenon. Finally, there are no direct randomised clinical studies that have specifically addressed the role of ATIs; direct comparisons are lacking between ATI-free diet and ATI containing diet, while controlling gluten and FODMAP content of diets. Comparing a gluten free diet to the habitual diet does not directly address the ATI-question because it has become evident that a gluten free diet is not only lower in its gluten content but also in the levels of FODMAPs

It is of interest that when wheat is fermented for a long time, such as in the traditional sourdough method, microbial proteases cleave gluten and other protein molecules into smaller units. Several studies have confirmed the hydrolysis of proteins during the fermentation process (Loponen et al. 2007, Rizello et al. 2007, De Angelis et al. 2009, Di Cagno et al. 2010). If hydrolysis is extensive enough, it is possible that it might inactivate the ATIs. However, ATIs are very resistant to heat and human digestive enzymes (Junker et al. 2012, Zevallos et al. 2017), and therefore it remains to be seen if ATIs truly could be hydrolysed into inactive peptides and amino acids during baking methods that exploit long fermentation times. Furthermore, there is evidence that fructans (FODMAPs) in bread dough are also hydrolysed during the long fermentation process either by using sourdough or a long 4-hour yeast baking method (Chavan and Chavan 2011, Zigler et al. 2016). Consequently, the fructan content of sourdough breads might be lower than that of yeast breads (Monash University low FODMAP application).

In summary, the available studies suggest that NCGS/NCWS can be verified by blinded tests only in a minority of people with self-diagnosed NCGS/NCWS. It is likely that a large part of NCGS/NWGS is explained by FODMAPs, which per se, suggests that patients with NCGS/NCWS might be on the continuum of IBS and not represent a separate clinical entity. The role of ATIs can only be ascertained in randomised clinical trials comparing an ATI-free diet to an ATI-containing diet while controlling for other confounding factors.

2.4 Dietary treatment of IBS

People with IBS commonly associate their symptoms with food; the perceived problematic foods include milk and other dairy products, legumes and pulses, cruciferous vegetables, some fruits, and grains, especially wheat and rye (Simrén et al. 2001, Gibson 2017). The scientific literature has expanded substantially during the past two decades with regards to diet and IBS; consequently, diet has now a central role in the treatment of IBS in many parts of the world.

No robust evidence exists that the amount or proportion of macronutrients (protein, carbohydrates and fat) would have a major effect in IBS although on the basis of mechanistic studies, fat might be an exception. There are laboratory studies indicating that the duodenal infusion of lipids can inhibit small bowel motility and impair intestinal gas clearance, inducing gas retention and bloating (Passos et al. 2005, Salvioli et al. 2006). Duodenal lipids also enhance colorectal hypersensitivity (Caldarella et al. 2005, Simrén et al. 2007). These types of studies should be always interpreted with care especially since the evidence associating dietary fat intake to IBS is very limited; results derived from experiments conducted under artificial conditions and extreme feeding practices are seldom transferable to the clinical setting as such. One acute study showed that a meal high in dairy fat (cream) and calories (800 kcal) increased acutely visceral sensitivity in patients with IBS and these might provoke feelings of abdominal pain and distension after the consumption of fat (Törnblom et al. 2014). The partial exclusion of poorly absorbable carbohydrates has long been part of dietary strategy in IBS, even although there were no randomised studies before the era of low-FODMAP diets (Dapoigny et al. 2003).

2.4.1 Fermentable carbohydrates

Australian researchers from Monash University introduced the concept of Fermentable Oligo-, Di-, Monosaccharides and Polyols (FODMAPs) (Gibson 2017). FODMAPs are simple carbohydrates which are not absorbed in the small intestine but rapidly and extensively fermented in the upper part of the colon. In the presence of impaired handling of intestinal gas, abnormalities in bowel motility and visceral sensitivity, it is the rapid fermentation of these FODMAPs that is postulated to trigger the symptoms of IBS (Gibson 2017). It is now widely accepted, based on randomised and mechanistic studies which will be detailed in the following chapters, that FODMAPs can be viewed as one group of culprits behind the aggravation of symptoms in IBS (Staudacher and Whelan 2017).

Background and theory

There are several review articles available that explain the theory and rationale of a low FODMAP diet in great detail (Gibson and Shepherd 2010, Gibson et al. 2015, Staudacher et al. 2015, Varney et al. 2017, Staudacher and Whelan 2017,). In brief, a low-FODMAP diet is a holistic dietary pattern where the amount of poorly absorbable carbohydrates (FODMAPs) that are also prone to bacterial fermentation in colon is substantially reduced. The mechanisms through which FODMAPs may cause symptoms in susceptible

individuals involve osmosis and fermentation (Staudacher and Whelan 2017). The aim of a low FODMAP diet is not to reduce the quantity of dietary FODMAPs to zero, in contrast to a gluten free diet where total restriction of gluten is required. Typically, after adoption of a low FODMAP diet, individuals still have a FODMAP intake of approximately 15-60% of their habitual diet (Ong et al. 2010, Staudacher et al. 2012, Halmos et al. 2014). FODMAPs are naturally occurring in many grains, legumes, fruits,vegetables and in some dairy and industrially processed foods such as prebiotic containing yogurts, fructose-sweetened carbonated drinks, protein bars and chewing gums. Edible fats and animal-protein rich foods are usually low or free from FODMAPs (Gibson and Shepherd 2010). Lactose containing dairy products are an exception; they are high in FODMAPs but it is noteworthy that lactose needs to be avoided only in the co-presence of lactose intolerance (Gibson and Shepherd 2010). Lactose is well absorbed as long as there is the presence of normal lactase enzyme activity; relevant quantities of lactose are thought to reach colon only if the activity of lactase is low or absent.

Table 2 details the FODMAPs groups and their dietary sources. The basis of a low-FODMAP diet is to switch from a high FODMAP food to a low FODMAP alternative from the same category; for example rye bread can be replaced by oat bread. High FODMAP foods can aggravate the symptoms in at least two ways: a) they cause increased retention of water in the distal small intestine and b) increase gas production in the colon due to the extensive and rapid fermentation of these short chain carbohydrates.

Table 2. Categorising of FODMAPs, their typical dietary sources and options for substitution. Note! The list of dietary sources is not exhaustive*.

FODMAP group	Biochemical entities	Main dietary sources (examples)	Low FODMAP options
Oligosacchrides	Fructo-oligosachharide (FOS)/ inulin (fructans), galacto-oligosaccharide (GOS)	Wheat, rye, barley, beans, onion, garlic, soy, some artificial sweetener powders	Oats, quinoa, rice, teff, amaranth, tofu, potate, low-FODMAP gluten free pasta
Disaccharides	Lactose (only when lactose intolerance is present)	Lactose containing milk, unripened cheese and yogurt	Orange, clementine, kiwi, honey dough melon, oat milk, some soy milk products, low-lactose/lactose free milk dairy products
Monosaccharides	Fructose (only when in excess of glucose)	Apple, pear, watermelon, orange juice, apple juice, mango, honey, high-fructose corn syrup	Orange, clementine, kiwi, honey dough melon, grapes, pineapple
Polyols	Isomalt, lactitol, mannitol, maltitol, sorbitol and xylitol (excluding erythritol)	Sugar free chewing gum/dents, plum, peach, apricot, nectarine, avocado, mushrooms, cashew nuts, pistachio nuts	Orange, clementine kiwi, blueberries, strawberries, lingon berries, raspberries, walnuts, peanuts, pecan nuts, pumpkin seeds, linseeds

*) See Monash University Low FODMAP Diet™ Application for further details

Arabinoxylan-oligosaccharides

It is noteworthy that the list of FODMAPs does not contain all of the rapidly and extensively fermentable carbohydrates that are found in food items, for example, researchers have identified arabinoxylooligosaccharide (AXOS) (Cloetens et al. 2008, Scarpellini et al. 2018) which is derived via the fermentation of arabinoxylan. Arabinoxylan in turn is a prominent fibre in rye, wheat and in barley (Frølich et al. 2013).

AXOS is produced into rye or wheat products via the use of industrial enzymes or via yeast/sourdough fermentation. Choetens et al. (2010) have estimated that refined wheat bread made with exogenously added xylanases according to current commercial practices

contains 1·32 g AXOS per 100 g dry matter while rye bread contains 2·64 g AXOS per 100 g dry matter. Beer is a product which has a particularly high AXOS content (Broekaert et al. 2011). However, there is virtually nothing in the literature about the presence of AXOS in grain products. There are no clinical studies on AXOS in IBS, but given the abundance of AXOS in bread products, and its extensive fermentation in colon, the role of AXOS in IBS should be addressed in the future studies.

Randomised studies on the efficacy of low FODMAP diet for IBS symptoms

There are at least two published meta-analyses of randomised controlled trials in IBS with the first being published by Khan et al. (2015) and the second by Marsh et al. (2016). Khan's meta-analysis included four randomised studies and it concluded that a low FODMAP diet is a relatively simple and safe treatment to reduce the symptoms of IBS. On the basis of their meta-analysis, the authors added that the number needed to treat (NNT) was 2.2 with respect to the overall improvement of symptoms (95 % confidence interval: 1.89–2.51). An NNT value of 2.2 can be considered as highly effective when compared to other chronic pain conditions such as neuropathic pain in adults; typical NNTs for the drugs of choice in neuropathic pain vary in a range 3.6-7.7 (Finnerup et al. 2015).

Marsh et al. (2016) analysed six randomised studies and sixteen observational interventions. In the meta-analysis that concccentrated on the randomised trials, IBS-SSS was reduced (OR 0.44, 95 % CI 0.25-0.76) and the quality of life was improved (OR 1.84, 95 % CI 1.12-3.03). Furthermore, adherence to a low FODMAP diet was found to significantly reduce symptom severity for abdominal pain (OR 1.81, 95 % CI 1.13-2.88;), bloating (OR 1.75, 95 % CI 1.07-2.87) and overall symptoms (OR 1.81, 95 % CI 1.11-2.95). In addition, the sixteen non-randomised studies lasting 1-35 months found very similar perhaps even more, beneficial effects. Since the appearance of the meta-analysis of Marsh et al. (2016), at least five randomised studies have been published on a holistic low FODMAP diet (Harvie et al. 2017, Vincenzi et al. 2017, Esweran et al. 2017, Staudacher et al. 2017b, Pedersen et al. 2017). In all of these new studies, a low FODMAP diet reduced overall symptoms vs. control treatment. It is interesting that a low FODMAP diet has been also applied in IBD, and a recent systematic review showed that a low FODMAP diet could reduce IBS like symptoms (abdominal pain, bloating, fatigue and diarrhoea) also in IBD which is an overt inflammatory condition (Zhan et al. 2018). The currently published randomised studies among adults with IBS are illustrated in Table 3.

Table 3. Features of the published randomised controlled studies on holistic low FODMAP diet among adults with IBS.

Study (Year), Country	Duration	Randomised (n)	Main effect on symptom control
Low FODMAP vs. Other kind of IBS Diet*			
Eswaran et al. (2016), USA	4 weeks	92	Low FODMAP diet resulted in a higher proportion of abdominal pain responders (51% vs. 23%, P=0.008); no difference in adequate relief
Böhn et al. (2015), Sweden	4 weeks	75	No difference in IBS-SSS
Staudacher et al. (2012), United Kingdom	4 weeks	41	More patients in the low FODMAP group reported adequate control of symptoms compared with controls (68% vs. 23%; P = 0.005).
Low FODMAP vs. Medium/High FODMAP Diets			
Staudacher et al. (2017), United Kingdom	4 weeks	102	IBS-SSS was lower for patients on the low FODMAP diet than on the sham** diet (173 vs. 224; P = .001)
Harvie et al. (2017), New Zealand	3 months	50	IBS-SSS was reduced more in the low FODMAP group compared with the high FODMAP group (-146.8 points vs. -43.2 points from baseline; P<0.0002)
McIntosh et al. (2016), Canada	3 weeks	40	IBS-SSS was reduced more in the low FODMAP group compared with the high FODMAP group (72% vs. 21%; P<0.009)
Halmos et al. (2014), Australia	3 weeks	30	Sum of IBS symptoms on VAS was reduced more in the low FODMAP group compared with the high FODMAP group (44.9 mm vs. 22.8 mm; p<0.001)
Pedersen et al. (2014), Denmark	6 weeks	82	IBS-SSS was reduced more in the low FODMAP group compared with the high FODMAP group (-133 points vs. -34 points from baseline; P<0.01)
Ong et al. (2010), Australia	11 days	15	Sum of six IBS symptoms on scale 0-4/each was higher on the high FODMAP diet than when consuming the low FODMAP diet (median 6 vs. 2; P = 0.002)

*) IBS diet of National Institute of Clinical Excellence or Swedish traditional IBS diet; **) In the sham diet group, participants were given false information on the content of FODMAPs in food items; this can be considered as a placebo group

Despite the fact that many randomised studies have shown efficacy in IBS, some methodological challenges are inevitable in dietary interventions and these have led some researchers to criticise the current evidence base for the benefits of a low FODMAP diet in IBS (Kroogsgaard et al. 2017). In their systematic review of nine randomised studies, these authors pointed out that low FODMAP studies tended to have a short duration, i.e. usually less than two months, blinding of the treatment was double-blind only in one study and

control diets were often habitual diets or commonly consumed national average diets. When the control group is not adequately active, i.e. there is no change towards a theoretically healthier diet, the placebo effect will favor the low FODMAP group rather than usual or national average diet. Italian researchers published another critical paper (Catassi et al. 2017). They argued that the criteria for inclusion of foods in the FODMAP list are not well defined. They also added that the drastic reduction of FODMAP intake could possibly also exert negative physiological consequences on the intestinal microbiota and colonocyte metabolism and even on the nutritional status of subjects with IBS.

Kroogsgaard et al. (2017) did not include in their systematic review the tightly double-blinded controlled studies on individual FODMAPs; in other words, Kroogsgaar's review paints only a partial picture of the evidence base. The following well-controlled studies were excluded from Kroogsgaard's review and therefore deserve attention. Shepherd et al. (2006) conducted a double-blind cross-over study on 25 IBS patients. When comparing a fructose drink, a fructan drink or their combination in a drink to placebo (glucose) drink, 70% of patients receiving fructose, 77% receiving fructans, and 79% receiving the combination drink reported that their symptoms were not adequately controlled, compared with 14% receiving glucose. This endpoint "not adequately controlled" was their answer to the question, "Were your symptoms adequately controlled in this phase?". In a Norwegian study, IBS patients reported clear worsening of symptoms when consuming a FOS drink for ten days while placebo drink (maltodextrin) did not cause any worsening of symptoms (Hustoft et al. 2016). In both the Hustoft and Shepherd investigations, the background diet was a low-FODMAP diet. In addition, in a third placebo-controlled study, FOS caused a worsening of functional GI symptoms while neither gluten nor placebo evoked any deterioration in people with a subjective gluten sensitivity (Skodje et al. 2018).

Staudacher et al. (2017 b) attempted to tackle the issue of a placebo effect. They were the first to report the results of a sham diet including 104 participants with IBS. This is the only study that sought to blind the low FODMAP diet advice given to the patients. Half of the participants were randomised to a low FODMAP diet and the other half to a sham diet. The participants who were randomised to the sham diet received an identical amount of attention as those on low FODMAP diet, but also received wrong advice on the low FODMAP diet. For example, the food list of the sham diet stated that rice and oats would not be suitable choices (even although they actually are low in FODMAPs) whereas wheat and barley would be suitable (even although they are high in FODMAPs). The participants were aware of the design of the study before entering the study; this is important in studies with a sham design in order to comply with the ethical norms of research. The results of this study showed that a low FODMAP diet was more effective in reducing GI symptoms than a sham diet. The total mean IBS-SSS was significantly lower for patients on a low FODMAP diet. One potential weakness of this particular design is that the blinding may have been lost during the treatment because in the era of an open internet, participants on the sham diets may have sought out information on an authentic low FODMAP diet, and thereby become aware of the true nature of their diet. This highlights the real-world challenges in blinding dietary treatments.

Finally, a recent intragastric FODMAP infusion test demonstrated that a fructan infusion provoked the prompt generation of GI symptoms in IBS and elevated the intragastric pressure when compared to healthy subjects (Masey et al. 2017). This is an intriguing finding as the symptoms could not be related to colonic fermentation because the symptoms appeared already within 30-60 minutes after infusion.

To summarise, it seems that FODMAPs, or at least fructans such as FOS, cause symptom aggravation in IBS patients even in double-blind conditions.

Effects on microbiota

It has become increasingly evident that there needs to be a holistic understanding of the role of FODMAP on an individual's health. FODMAP researchers in King's College, London and Monash University, who are pioneers of the FODMAP concept, recommend that more research should be conducted in this area (Staudacher and Whelan 2017, Hill et al. 2017). The main reason for the increased caution is the potential negative effect of a low FODMAP diet on intestinal microbiota. Several clinical studies have shown that a low FODMAP diet tends to lead to a reduction in the amount of beneficial intestinal bacteria, i.e. the relative of absolute abundance of bifidobacteria, lactobacilli, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* is claimed to be lower during the low FODMAP diet (Staudacher et al. 2012, Chumpitazi et al. 2014, Halmos et al. 2015, Hustoft et al. 2016, McIntosh et al. 2016, Staudacher et al. 2017b, Bennett et al. 2018) Furthermore, the diversity of the microbiota might also be reduced (Bennett et al. 2018). The most consistent finding in these studies has been the decreased abundance of intestinal bifidobacteria. These dysbiotic changes might exert an overall negative effect on health, especially because people with IBS tend to have a decreased level of these bacteria even before starting the FODMAP diet (Liu et al. 2017). Due to the above mentioned reasons, a low FODMAP diet is not intended for life but for a short duration of 1-3 months after which a slow re-introduction of FODMAPs should be pursued within the boundaries of individual tolerance (Tuck and Barrett 2017, Harvie et al. 2017). One final note, the role of the observed changes in microbiota for overall health is still speculative because there are no long-term prospective studies investigating a low FODMAP diet. The effect of low FODMAP diet on long-term health can be only tested in long-term clinical trials; it is too early to draw any definitive conclusions on the role of microbial changes.

Effects in immune system and permeability

In this context, it is interesting that exclusive enteral nutrition, which is often low in FODMAPs, causes dramatic negative changes in the intestinal microbiota but also induces remission in patients suffering from difficult-to-treat Crohn's disease (MacLellan et al. 2017). Conventionally, dietary studies in IBS have measured mainly symptoms and occasionally hydrogen/methane excretion and/or microbial changes. Recently, studies have started to emerge suggesting that a low FODMAP diet could reduce low grade inflammation or immune activation in people with IBS (McIntosh et al. 2016, Hustoft et al. 2017). McIntosh et al. found that the introduction of a low FODMAP diet reduced the levels of a urinary metabolite of histamine by 85%, and Hustoft et al. (2017) demonstrated

that a low FODMAP diet reduced levels of two pro-inflammatory markers, IL-6 and IL-8, in blood. The former finding suggested that mast cell induced activation of the immune system was reduced during the low FODMAP diet, and the latter study suggested that a low FODMAP diet may have systemic anti-inflammatory properties in people with IBS. These are potentially important findings because pro-inflammatory markers such as IL-6 are associated with an increased risk of coronary heart disease (IL6R Genetics Consortium Emerging Risk Factors Collaboration 2016).

A Danish randomised study was performed among IBD patients in remission or with mild symptoms (Pedersen et al. 2017). A statistically significant reduction in the Simple Clinical Colitis Index was observed for the low FODMAP diet in patients with ulcerative colitis (UC) but not in Crohn's disease, IBS like symptoms were also reduced by low FODMAP diet in both UC and Crohn's disease patients. However, despite these encouraging findings on the benefits of a low FODMAP diet, many more studies will be needed to clarify the anti-inflammatory potential of a low FODMAP diet in IBS. Whatever is the case, one can argue that a low FODMAP diet has potentially beneficial anti-inflammatory effects in IBS but the downside is the possibility of dysbiotic changes in gut microbiota. It needs to be clarified which of these phenomena is more important for overall health.

To summarise, a low FODMAP diet is effective in reducing the symptoms of IBS in most patients. Its long-term effects are still unknown and therefore long-term randomised studies are needed; currently strict adherence to a low FODMAP diet is recommended for some months only. A low FODMAP diet reduces the abundance of beneficial bacteria in gut but in contrast, it may also reduce elevated immune activation in IBS. The FODMAP concept does not cover all rapidly fermentable carbohydrates such as AXOS which also might cause symptoms in IBS patients.

2.4.2 Grains containing gluten and amylase trypsin inhibitors

Gluten-containing grains are often suspected to be behind IBS like GI symptoms. In short, the literature strongly suggests that in the most of the cases of subjective NCGS or NCWS, gluten or ATIs per se are not the "culprit" behind the symptom aggravation experienced when consuming gluten-containing grains such as wheat, rye or barley. It is claimed that some other compound, not the gluten in grains, must be behind the symptoms.

In an interesting study, Uhde et al. (2016) showed that a six-month gluten free diet improved symptom control and reduced several inflammatory/permeability markers i.e. IL-8, LBP, flagellin and FABP2. However, because there was no proper control group, it is not known if the observed effect was due to gluten withdrawal, to a reduced intake of FODMAPs, or ATIs, or to some unknown factor. One randomised study showed that intestinal permeability in the small intestine but not in the large bowel was decreased in IBS-D patients consuming a gluten free diet and in parallel, stool consistency was improved (Vazquez-Roque et al. 2013). This is the only existing randomised study that has explored the effects of a holistic gluten free diet in IBS. Previously another six-month non-

controlled study had shown that people with IBS-D gained benefits from a gluten free diet (Wanschaffe et al. 2007).

Avoidance of wheat, rye and barley leads to a decreased intake not only of gluten but also FODMAPs and ATIs. Table 4 illustrates the amount of gluten, FODMAPs and ATIs in different grain products. As the table demonstrates, when people are placed on a gluten free diet, it is likely that their intake of FODMAPs and ATIs will be reduced in addition to the decrease in their gluten intake. Currently, there are no direct comparison studies between FODMAPs and gluten/ATIs, that would reveal whether it is FODMAPs or gluten or ATIs that are responsible for the increase of GI symptoms in NCWS and/or in IBS.

Table 4. Comparison of the gluten, fructans and ATI contents of some grain products. Data is compiled from the following sources: Biesiekierski et al. 2011 a, Frølich et al. 2013 and Zevallos et al. 2016.

	Gluten	FODMAPs (Fructans mainly)	ATIs
Wheat	High	High	High
Rye	High	High	High
Barley	High	High	High
Oats	Non-existent	Low	Low
Rice	Non-existent	Low	Low

An innovative confocal endomicroscopy study showed that in 13 out of a total of 36 IBS patients with a suspected unspecified food intolerance, exposure to wheat antigen mixture caused immediate breaks, increased intervillous spaces, and increases in the numbers of intraepithelial lymphocytes in the intestinal mucosa but none of the subjects reacted to wheat in the control group (Fritscher-Ravens et al. 2014). Patients with coeliac disease and food allergies were excluded. These changes were associated with patient responses to exclusion diets in the open follow-up study. These data suggest that not all symptoms are related to colonic fermentation but instead the food itself can exert immediate local effects in gut.

Robert Spiller and co-workers have conducted a series of interesting functional MRI studies in humans. One of these studies compared the effect of wheat and rice on GI motility in healthy subjects (Marciani et al. 2013). They found out that whole wheat bread forms a homogeneous bolus in the stomach, which inhibits gastric sieving and hence empties slower than the equicaloric rice meal. In addition, the wheat meal remained in its postprandial form in the small intestine and decreased the small intestine water content. In another MRI study, the same research group has shown that people with IBS-D or IBS-M seem to have a constricted small bowel when compared to healthy subjects (Lam et al. 2017). These data can be interpreted that wheat has negative digestive effects unrelated to colonic fermentation, in especially in the upper GI tract. However, the biochemical mechanism behind slower gastric emptying and decreased small bowel water remains to be clarified.

Many grains contain relatively high amounts of phytic acid (Gupta et al. 2015) but no studies exist on the role of phytic acid in functional gastrointestinal disorders such as NCGS/NCWS or IBS. In theory, phytic acid might evoke symptoms by binding minerals and thus exposing the colon to an increased level of unabsorbed minerals such calcium and magnesium, which in turn might cause irritation of epithelium. There is evidence from animal experiments that increasing amounts of phytic acids in the diet may induce beneficial effects on long-term gut health; the production of short-chain fatty acids was increased and a reduction in systemic inflammation was observed when mice were fed with a diet high in phytic acid (Okazaki and Katayama 2014).

Grains also contain lectins which serve as defense mechanisms against other plants and fungi (De Punder and Pruimboom 2014). No clinical studies exist on the role of lectins in NCGS/NCWS or IBS but preliminary animal and in vitro studies suggest that wheat agglutinin, lectin in wheat, may cause inflammation or provoke histamine release (De Punder and Pruimboom 2014).

To conclude, gluten free grains also have lower FODMAP and ATI concentrations. It is not clear if it is the combination of both FODMAPs and gluten/ATI that is required to trigger symptoms in IBS or if one component is more dominant in symptom generation. Furthermore, the role of AXOS in grains is not well understood and has been poorly studied. MRI and confocal endomicroscopy studies suggest that some of the symptoms can appear quickly, within minutes or a couple of hours, from consumption of the culprit food, and therefore cannot be directly related to colonic processes such as fermentation.

2.4.3 Dairy

Lactose is a disaccharide present in milk consisting of glucose and galactose. Lactose intolerance is a very common condition caused by lactase deficiency. The global prevalence estimate of lactose intolerance is 68%, ranging from 28% in Europe to 70% in the Middle East; the Finnish prevalence is 19% (Storhaug et al. 2017). Lactase deficiency results in increased lactose levels entering the colon where bacteria ferment this sugar. Increased fermentative processes may cause gas problems and symptom aggravation especially in the presence of functional gastrointestinal disorders (Gibson and Shepherd 2010). Avoidance of lactose is not necessary during a low FODMAP diet if the patient does not have lactase deficiency.

In addition to lactose, other components, such as proteins or fat globules, present in dairy products, or introduced during their processing could cause symptoms in IBS, at least in theory. Paajanen et al. (2003) and Korpela et al. (2005) performed a series of small scale studies in patients with apparent milk intolerance. Patients were not pre-screened for IBS before entering the studies and thus it is not known if patients had IBS or not. Paajanen et al. (2003) undertook five-day challenge tests and claimed that patients tolerated homogenised and unhomogenised milk similarly; no difference in tolerance could be observed in any of three tests performed in two separate studies. In a 4-day randomised study, Korpela et al. (2005) demonstrated that homogenization of milk had no discernible

effects on symptoms in people with lactose intolerance when homogenised and non-homogenised milk were compared.

There are some small experiments that suggest that milk proteins together with lactose or other milk components can trigger symptoms. However, the limitations of these studies must be acknowledged; they are small, typically have a very short duration (acute experimental studies) and have been performed in subsets of functional gastrointestinal disorders. A dairy free diet has never been adequately tested in IBS in a randomised controlled study. A large Italian study reported that 22.4% of IBS patients who were subjectively intolerant to wheat also reacted negatively (experienced GI symptoms) to milk protein (casein and whey) pills in a blinded two-week test (Caroccio et al. 2012). Very much against expectations, the authors reported that none of the 904 patients reacted to placebo (xylose). It must be noted that this study was not designed to test milk protein intolerance but instead concentrated on wheat intolerance.

In small acute studies conducted in patients with subjective-based intolerance to cows' milk, the consumption of milk has produced increased immune activation in the intestinal epithelium and/or symptom aggravation with no such effect being detected in healthy controls (Bengtsson et al. 1997, Pelto et al. 1998, Kristjánsson et al. 2007). Some authors have also speculated that the currently common variant of milk casein, so called A1-casein, would be more pro-inflammatory than the more ancient A2-casein predominant milk. There are two randomised studies that have examined this topic; neither of them found differences in tolerance of the two milk products in subjects with subjective cow milk intolerance (Ho et al. 2014, Jianqin et al. 2015).

In a Finnish study, patients with subjective feelings of improved tolerance of milk when abroad were studied (Paajanen et al. 2004). As there was no rational explanation for the reports of improved milk tolerance when abroad, the authors speculated that people with this kind of experience might be especially sensitive to indigestible carbohydrates such as polyols or fructans. By performing an acute meal test, the authors concluded that xylitol and rye bread, which are high in fructans, cause more GI symptoms in people with experiences of improved tolerance of milk when abroad in comparison to those who experience no differences irrespective of where the milk is consumed. As such, this study can be viewed as one of the first studies paving the way to the FODMAP concept. Another Finnish randomised acute meal study reported aggravation of GI symptoms in people with subjective cow milk intolerance vs. healthy controls when consuming fructo-oligosaccharides or lactulose (not lactose) (Teuri et al. 1999).

In the previously mentioned confocal endomicroscopy study (Fritscher-Ravens et al. 2014) in 9 out of 26 IBS patients with suspected food intolerance, exposure to a milk antigen mixture caused immediate breaks, increased intervillous spaces, and increased intraepithelial lymphocytes in the intestinal mucosa while it caused no such effects in any of the controls (Fritscher-Ravens et al. 2014). When foods that had caused the epithelial breaks were excluded from the diet, the gastrointestinal symptoms were reduced in the open 4-week follow up study.

In summary, these data suggest that milk or dairy per se could cause a worsening of GI symptoms and/or activation of immune system at the level of gut epithelium in a small subset of people with functional gastrointestinal disorders. The studies have methodological weaknesses and robust studies are lacking. Furthermore, although some people may consider that dairy products are the sole triggers of their symptoms, in reality, it is the total amount of FODMAPs in the diet that is truly responsible for their symptoms.

2.4.4 Other dietary approaches

In a Swedish randomised study, Böhn et al. (2015) compared a conventional IBS diet to a low FODMAP diet but detected no differences in symptoms. It must be noted that the traditional IBS diet incorporated a partial FODMAP restriction because certain foods such as onions, legumes and chewing gum were to be avoided. In addition, soda, coffee, fatty foods and spicy foods were restricted and consumption of fibre was encouraged in the traditional diet. It is noteworthy that this was the first study (Böhn et al. 2015) to directly compare a traditional IBS diet to any other diet (control) in a randomised setting. The fact that a traditional IBS diet had never before been tested in a randomised trial underlines that fact that progress in dietary management has truly taken off only during the past decade or so.

A low carbohydrate diet reduced also GI symptoms in a non-controlled intervention; all patients had IBS-D (Austin et al. 2009) but no randomized studies exist investigating a low carbohydrate diet. A recent study compared a specific carbohydrate diet, commonly known as the SCD diet for IBD, to a low FODMAP diet in subjects with IBS; the low FODMAP diet resulted in less bloating than the SCD.

Finally, a well-controlled study was performed in patients with IgG antibodies against several food items (Atkinson et al. 2004). An individually tailored exclusion diet resulted in a 10% greater reduction in the symptom score than the sham diet. The global rating also significantly improved in the exclusion diet group as a whole ($p=0.048$, NNT=9) but not in the sham diet group. Unfortunately, details and formulation of the sham diet were not provided. The authors concluded “Food elimination based on IgG antibodies may be effective in reducing IBS symptoms and is worthy of further biomedical research”. Similarly, a recent study also found out that an individualised diet guided by leucocyte activation tests reduced symptoms compared to a sham-guided diet (Ali et al. 2017). Overall, there is a paucity of randomised studies regarding dietary patterns other than a low FODMAP diet.

2.4.5 Fibre

People with IBS have commonly gastrointestinal dysmotility, and more than half of the IBS patients have either constipation chronically (IBS-C) or periodically (IBS-M). Therefore, it

has been a long tradition to recommend an increased amount of dietary fibre in IBS (Manning et al. 1979). However, recent systematic reviews and critical reviews of randomised studies have highlighted the importance of choosing the right type of fibre both in people with IBS and in healthy controls; i.e. it is not only the total fibre intake that is important but also about the quality of fibre (Nagarajan et al. 2015, Christodoulides et al. 2016, McRorie et al.2016).

One common recommendation across the medical literature is to recommend soluble fibre in IBS (Moayyedi et al. 2014, Nagarajan et al. 2015). However, these data are overwhelmingly based on studies on psyllium (isphagula husk, plantago ovata) (Moayyedi et al. 2014, Nagarajan et al. 2015). FOS, inulin and GOS are also soluble fibres but in addition, they are high in FODMAPs and prone to cause worsening of IBS symptoms as shown by Hustoft et al. (2016), Shepherd et al. (2008) and Major et al. (2017). Furthermore, these soluble fibre preparations do not even relieve constipation, at least according to recent systematic reviews of randomised studies (Christodoulides et al. 2016, McRorie et al.2016).

The advantage of fructans and GOS is that they are prebiotic (Macfarlane et al. 2008); therefore they have some potential to correct the dysbiotic changes found in the microbiota of IBS patients. However, it is unclear how this would be possible without triggering of IBS symptoms. In one randomised study, a small dose of GOS actually improved most of the IBS symptoms; GOS also stimulated the amount of bifidobacteria (Silk et al. 2009). It remains to be seen if a cautious, incremental, increase in the consumption of fructans/GOS lasting many weeks could possibly allow consumption of these prebiotic substances without causing an aggravation of IBS symptoms. Currently there is a discrepancy between the study findings of Silk et al. (2009) vs. Hustoft et al. (2016), Shepherd et al. (2008) and Major et al. (2017) as mentioned above. Indeed, Hertzler and Savaiano (1997) have shown that the microbiota seems to adapt to an increased lactose intake by reducing the fermentation rate. At the same time, lactose tolerance was significantly increased in people with lactose intolerance. In healthy subjects, GOS caused microbial adaptation and reduced flatulence after weeks of administration when compared to the first day of administration (Mego et al. 2017).

More than a decade ago, it was a common practise to recommend wheat bran in IBS. Unfortunately, wheat bran is high in FODMAPs, i.e. fructans, and therefore its effects on IBS are unimpressive (Nagarajan et al. 2015); in many trials, wheat bran caused either worsening of bloating and/or abdominal pain and/or flatulence (Hotz et al. 1994, Snook and Shepherd 1994, Hebden et al. 2002). Rye bran has not been studied in IBS, but it must be acknowledged that similar to wheat bran, rye bran is also high in FODMAPs.

Psyllium is a poorly-fermentable, soluble fibre that is beneficial in relieving constipation both in healthy subjects and in people with IBS (Nagarajan et al. 2015, Christodoulides et al. 2016, McRorie et al.2016). It also seems to improve overall symptom control in IBS (Ford et al. 2014, Nagarajan et al. 2015) but it is unknown if other mechanisms apart from improved bowel habits play any role in overall symptom reduction. Psyllium is recommended for IBS by the American College of Gastroenterology (Ford et al. 2014).

There is some evidence that linseeds might be helpful; but overall, the evidence is still unconvincing (Nagarajan et al. 2015). Only two studies have shown that ground linseed might improve some of the symptoms of IBS (Cockerell et al. 2012, Tarpila et al. 2003). Partially hydrolysed guar gum is another type of soluble fibre, which may help in IBS; preliminary studies revealed that it could alleviate symptoms of IBS albeit methodological weaknesses of the studies complicate the interpretation of the data (Parisi et al. 2002, Niv et al. 2016). Oat bran, which contains soluble fibre, so far has not been studied in IBS.

In conclusion, psyllium, partially hydrolysed guar gum and linseeds have been shown to reduce the symptoms associated with IBS. These fibres should be used as first-line treatments in IBS. Data on other soluble fibre supplements FOS, GOS, inulin and wheat bran have been mostly discouraging. Therefore, health care professionals should stop promoting the consumption of soluble fibre without precisely naming the actual fibre supplement. There is a need for more robust and innovative fibre studies in IBS, especially as add-on treatments to supplement a low FODMAP diet.

2.4.6 Drinks

Binge drinking is an irritant of the gut mucosa; it leads to a pro-inflammatory status characterised by increased levels of endotoxin in blood (Bala et al. 2014), and caution in the consumption of alcoholic beverages is recommended in IBS (McKenzie et al. 2012). Alcohol consumption has been associated with a worsening of GI symptoms among IBS and dyspepsia patients (Hadler et al. 2006, Reding et al. 2016). However, no clinical studies have been performed in IBS.

Carbonated drinks are often perceived as triggers of IBS symptoms (Ligaarden et al. 2012), but no clinical research has been performed in IBS. Coffee is low in FODMAPs but in some people, it can increase rectosigmoid motor activity within 4 min after ingestion. Its effects on the colon are found to be comparable to those of a 1000 kcal meal (Boekema et al. 1999) and therefore might not be suitable in IBS-D. No randomised controlled studies have been performed with tea or coffee in IBS. However, very large doses (2 L/day) of tea might cause constipation in healthy subjects (Højgaard 1981) and coffee can worsen upper GI symptoms (Brazer et al. 1995).

2.4.7 Spices and herbal products

Chili spice is low in FODMAPs but many patients associate their symptoms with chili or other hot spices (Simren et al. 2001, Esmaillzadeh et al. 2013). In an acute meal study, chili increased abdominal pain and burning sensations in patients with IBS-D (Gonlachanvit et al. 2009). However, a clinical six-week study suggested that if consumed for long enough, then chili could actually decrease the symptoms of IBS (Bortolotti and Porta 2001). However, the caveat of this study is that eight patients (34%) out of 23 patients had to stop

prematurely in the group receiving chili pills due to abdominal pain. Interestingly, IBS patients seem to undergo an upregulation of the so-called capsaicin receptor i.e. TRPV1, in their colonic mucosa (Akbar et al. 2008); this suggests that IBS patients might be more sensitive than healthy controls to the capsaicin present in chil. Turmeric, or its active ingredient curcumin, has only been studied in IBS in mixtures either with fennel oil or with curry and pomegranate (Lauche et al. 2016). In the former study but not in the latter, turmeric showed some efficacy over placebo. Aloe vera has been tested in three IBS studies but only in one did it demonstrate better efficacy vs. placebo (Davis et al. 2006, Hutchings et al. 2011, Størsrudet al. 2015).

The efficacy of enteric-coated peppermint oil capsules has been demonstrated in multiple randomised studies (Khanna et al. 2014). Its use is recommended in IBS by the American College of Gastroenterology (Ford et al. 2014). Peppermint oil possesses anti-inflammatory and spasmolytic effects (Harris 2016) and it seems to alleviate abdominal pain and cramps. However, it also seems to increase the occurrence of heartburn. A herbal preparation, STW 5 (containing iberis, peppermint, chamomile) was able to reduce IBS symptoms in one randomised study (Madisch et al. 2004); its efficacy has been well documented in upper GI symptoms (Melzer et al. 2004).

2.5 Enzymes

Many commonly available digestive enzymes are classified as nutritional supplements in Finland, even if some exceptions exist such as a preparation containing pancreatic enzymes (Creon®, Abbott Laboratories GmbH, Neustadt, German) which is available only on prescription. Some enzyme supplements may theoretically reduce either the pro-inflammatory potential of wheat proteins or the fructan/GOS content by acting as proteases i.e. they break down proteins into peptides and amino acids. Invertase and α-galactosidase hydrolyse fructans/GOS into monosaccharides. A recent randomised study showed that the α-galactosidase enzyme could reduce some IBS symptoms related to oligo-galactosaccharides (GOS) (Tuck et al. 2017) but an earlier study indicated that α-galactosidase did not confer any true benefit as a sole treatment option when background diet was not controlled (Hillilä et al. 2016). The data from these trials suggest that α-galactosidase may reduce symptoms caused by foods high in GOS such as beans, almonds or lentils but it is not able to hydrolyse their FODMAPs such as fructans, lactose or polyols. Grains are particularly high in fructans, not in GOS. As far as I known, invertase, which hydrolyses fructans, has not been tested in IBS.

2.6 Probiotics

It is noteworthy that many randomised studies have been performed with probiotics in IBS. Several meta-analyses of these studies have been published i.e. 35 randomised studies and 7406 participants (McKenzie et al. 2016). On the basis of 9 meta-analyses, McKenzie

et al. concluded that probiotics seemed to alleviate overall symptoms modestly vs. placebo; however, the effects have been inconsistent across the studies. A further challenge is that there are no studies comparing the efficacies of different probiotics. No recommendation can be drawn from the literature which specific strains of probiotics to use in IBS. In their dietary guidelines of IBS, British Dietetic Association recommends (McKenzie et al. 2016):

"Advise that probiotics are unlikely to provide substantial benefit to IBS symptoms. However, individuals choosing to try probiotics are advised to select one product at a time and monitor the effects. They should try it for a minimum of 4 weeks at the dose recommended by the manufacturer. For individuals with IBS, taking a probiotic product is considered safe in IBS."

The World Gastroenterology Organisation published their guideline on probiotics and prebiotics in March 2017. This report names the bacterial strains that have shown some effectiveness at least in one of the IBS-related parameters, and suggests that certain probiotics might reduce either individual symptoms or overall symptom control but the strength of the evidence was not considered as particularly strong.

Very recently, Staudacher et al. (2017) reported that a probiotic containing 8 different strains did not reduce symptoms in IBS when co-administered with a low-FODMAP diet but increased the numbers of Bifidobacterium species, as compared to placebo, when given at the same time with a low FODMAP diet.

2.7 Drugs

Many other factors influence the course of IBS in addition to dietary factors. Drug treatment is common practice especially in more severe cases of IBS (Chey et al. 2015): antidepressants and antispasmodics are used for abdominal pain and overall relief, prosecretory agents such linaclotide and lubiprostone for constipation and overall relief, the locally acting antibiotic, rifaximine, for bloating and overall relief and 5-HT3 receptor-antagonists, alosetron or ramosetrin, for diarrhoea and overall relief.

Camilleri and Boeckxstaens (2017) have recently reviewed upcoming and existing drug treatments against abdominal pain in IBS. These approaches include antispasmodics, antidepressants (tricyclic agents, selective serotonin reuptake inhibitors), 5-HT3 receptor antagonists (alosetron, ondansetron, ramosetron), a non-absorbed antibiotic (rifaximin), secretagogues (lubiprostone, linaclotide), μ-opioid receptor (OR) and κ-OR agonist, δ-OR antagonist (eluxadoline), a histamine H1 receptor antagonist (ebastine), a neurokinin-2 receptor antagonist (ibodutant) and GABAergic agents (gabapentin and pregabalin). Only three drugs (eluxadoline, linaklotide and rifaximin) are indicated for the treatment of IBS and available for clinical use in EU.

Drug therapy of IBS-D has recently been reviewed (Dothel et al. 2018). Administration of the 5-HT3 receptor antagonist, ramosetron, has demonstrated some promise in clinical

studies but is currently unavailable in the EU or USA. Eluxadoline, a μ- and κ-opioid receptor agonist and δ-opioid receptor antagonist that acts locally in the enteric nervous system and might also decrease the adverse effects on the central nervous system; it is marketed for IBS-D both in the EU and USA (Dothel et al. 2018). The official product information text of eluxadoline in EU warns about serious side effects; pancreatitis and spasm in the sphincter of Oddi can occur.

Linaklotide, a prokinetic drug, is currently available for pharmcological treatment of IBS-C in the EU and USA (Shah et al. 2018). Linaclotide belongs to the class of guanylate cyclase-C agonists; it seems to reduce constipation but also increases the incidence of diarrhea (Shah et al. 2018). It is also indicated in the treatment of idiopathic constipation.

Rifaximin, a locally acting antibiotic, has demonstrated efficacy against feelings of distension and overall well-being in gut but has not reduced the prevalence of other individual symptoms according to a meta-analysis of randomized studies (Li et al. 2016). The effects of this kind of antibiotic treatment on the gut health over the long term are far from clear; clinical studies have been shorter than 6 months (Li et al. 2016).

It is important to acknowledge that some medications used for conditions other than IBS may worsen the symptoms experienced by IBS patients. Chey et al. (2015) warned that antibiotics, antidepressants, antiparkinsonian drugs, antipsychotics, calcium-channel blockers, diuretics, metformin, opioids such as codeine and sympathomimetics would all be prone to cause symptom aggravation in IBS. In addition, some over-the-counter drugs (non-steroidal anti-inflammatory drugs) and supplements (iron, calcium, magnesium) are also potential triggers of the symptoms of IBS (Chey et al. 2015).

2.8 Exercise, sleep and psychological factors

Exercise and sleep patterns affect the symptoms of IBS. Physical exercise interventions have achieved a clear improvement in IBS symptoms when daily physical activity was increased (Villoria et al. 2006, Johanssen et al. 2011). It seems that already daily walking of 30 minutes has the potential to reduce the symptoms of IBS (Johannessen et al. 2015). On the day after a poor night's sleep, IBS patients seem to suffer from more severe symptoms (Buchanan et al. 2015) and a systematic review suggested that sleep disturbances were related to a higher risk of having IBS (Tu et al. 2017). However, more studies will be needed to clarify the role of sleep in IBS.

Finally, different kinds of psychosocial interventions have the potential to reduce symptoms in IBS. Gut-directed hypnotherapy, mindfulness and cognitive behavioural therapy are all claimed to be effective in reducing symptoms of IBS according to systematic reviews of the relevant literature (Peters et al. 2015, Asare et al. 2012).

2.9 Placebo and nocebo effect

As in any disorder where severity or the disorder state is measured by subjective evaluation, measurement of IBS status is also prone to a placebo effect. A recent systematic review demonstrated that the pooled mean placebo response rate in IBS was 41.4% and it does not seem to differ substantially between treatment modalities such as diet, drug treatment of psychosocial interventions (Flik et al. 2017). Similar placebo rates have been reported in systematic reviews examining migraine (Fernandes et al. 2008) and major depression (Walsh et al. 2002). Due to the very existence of placebo and nocebo effects, dietary studies should be well controlled and blinded whenever possible, and there is an increasing need to measure objective parameters in parallel to symptoms, i.e. hydrogen excretion, markers of immune activation and microbiota (Rao et al. 2015, Gibson et al. 2017, Molina-Infante and Carroccio 2017). As detailed in the text, this has been increasingly the case in many IBS studies published during the past 5 years. On the basis of randomised studies, it has been estimated that the nocebo effect is 3 times larger than gluten specific negative reactions in people with NCGS/NCWS.

2.10 Summary and hypotheses

The symptoms of IBS are often associated with the consumption of specific food items. Several intervention studies have shown that a low FODMAP diet, peppermint oil, psyllium fibre and possibly certain probiotics may reduce the symptoms in patients affected by IBS. In fact, it seems that in IBS no other dietary pattern has ever been studied as much as a low FODMAP diet. Nevertheless, there is still a paucity of *placebo* controlled FODMAP studies on food items naturally rich in FODMAPs. Furthermore, gluten sensitivity overlaps strongly with IBS and according to randomised controlled trials it is only seldom a truly gluten specific condition. It is more likely that the typical FODMAPs of grains, i.e. fructans as well as a nocebo effect and hypothetically ATIs are the actual causes of symptom aggrevation in individuals with gluten sensitivity. Randomised studies are needed especially to clarify the detailed role of ATIs in gluten sensitivity as no clinical studies have been performed on ATIs in gluten sensitivity.

The first hypothesis of this thesis was that the consumption of a rye bread lower in FODMAPs would cause less IBS symptoms, evoke favourable changes in intestinal function (colonic fermentation, pH, transit time or intestinal pressure) and not cause any negative effects on intestinal microbiota since the FODMAP content was lower than regular rye bread, which has a high fructan content. The second hypothesis was that the consumption of sourdough wheat bread with its lower levels of ATIs and fructans would reduce gastrointestinal symptoms and low grade inflammation in people with gluten sensitivity when compared to regular yeast leavened wheat bread which has a higher content of these compounds.

3 AIMS

The aims of this thesis work were as follows:

1. To determine whether low FODMAP rye bread reduces gastrointestinal symptoms and colonic fermentation among individuals with IBS when compared to regular rye bread in a four-week randomised trial (Study I)

2. To assess whether wheat bread baked in long fermentation process without yeast and additives, i.e. wheat bread lower in ATIs and FODMAPs, reduces symptoms when compared to regular wheat bread among individuals with non-coeliac wheat sensitivity and IBS. (Study II)

3. To study if low-FODMAP rye bread changes gastrointestinal pressure, transit time and pH differently from regular rye bread in an acute meal study in individuals with IBS (Study III)

4. To define the effects of low-FODMAP rye and regular rye bread on intestinal microbiota in individuals with IBS. (Study IV)

4 MATERIALS AND METHODS

As the methodologies applied in studies I-IV varied, the brackets after the subheadings describe in which studies a particular method was used.

4.1 Subjects and study designs (I-IV)

The study settings, subjects and interventions in each study are shown in Table 5 and the individual study designs in Figure 3.

Table 5. Characterisation of the study settings, interventions and subjects.

Study	Subjects	Number of subjects	Design	Intervention	Numbers of participants completing the study (%)	Duration
I	Free-living IBS subjects	87	Double blind, cross-over, randomised	Low FODMAP rye bread vs. regular rye bread	73 (84%)	4 weeks
II	Free-living IBS/NCWS subjects	26	Double blind, parallel groups, randomised	Yeast wheat bread vs. sourdough wheat bread	26 (100%)	7 days
III	IBS subjects in a laboratory	9	Double blind, cross-over, randomised	Low FODMAP rye bread vs. regular rye bread	7 (78%)	12 hours
IV	Free-living IBS subjects	50	Double blind, parallel groups, randomised	Low FODMAP rye bread vs. regular rye bread	50 (100%)	4 weeks

All the studies were performed as double blind randomised clinical studies. Papers I and IV are derived from the same study. Computer assisted randomisation was used.

Study candidates were pre-screened by nutritionists and those individuals meeting the inclusion criteria were subsequently referred to a screening consultation with a gastroenterologist to ensure their eligibility for participation. Blood count, sedimentation rate, transglutaminase antibodies for coeliac disease and thyroid function tests (P-tyreotropine and thyroxine) were done before the physician's screening visit if these tests had not already been performed within the past 12 months. Participants were recruited from the Helsinki Metropolitan area via the internet (pronutrionist.net) and through gastroenterologist appointments at Aava Medical Centre. Participants were pre-screened

by gastroenterologists before entering the studies in order to ensure that they met the inclusion criteria. All participants fulfilled Rome III criteria for IBS. In studies I and IV, only subjects with IBS-D, IBS-M and IBS-U were recruited. In studies II and III, all IBS subtypes were included. In addition, the participants in study II needed to have subjectively based poor tolerance of wheat. A further inclusion criterion for all studies was that participants needed to be 18-65 years old.

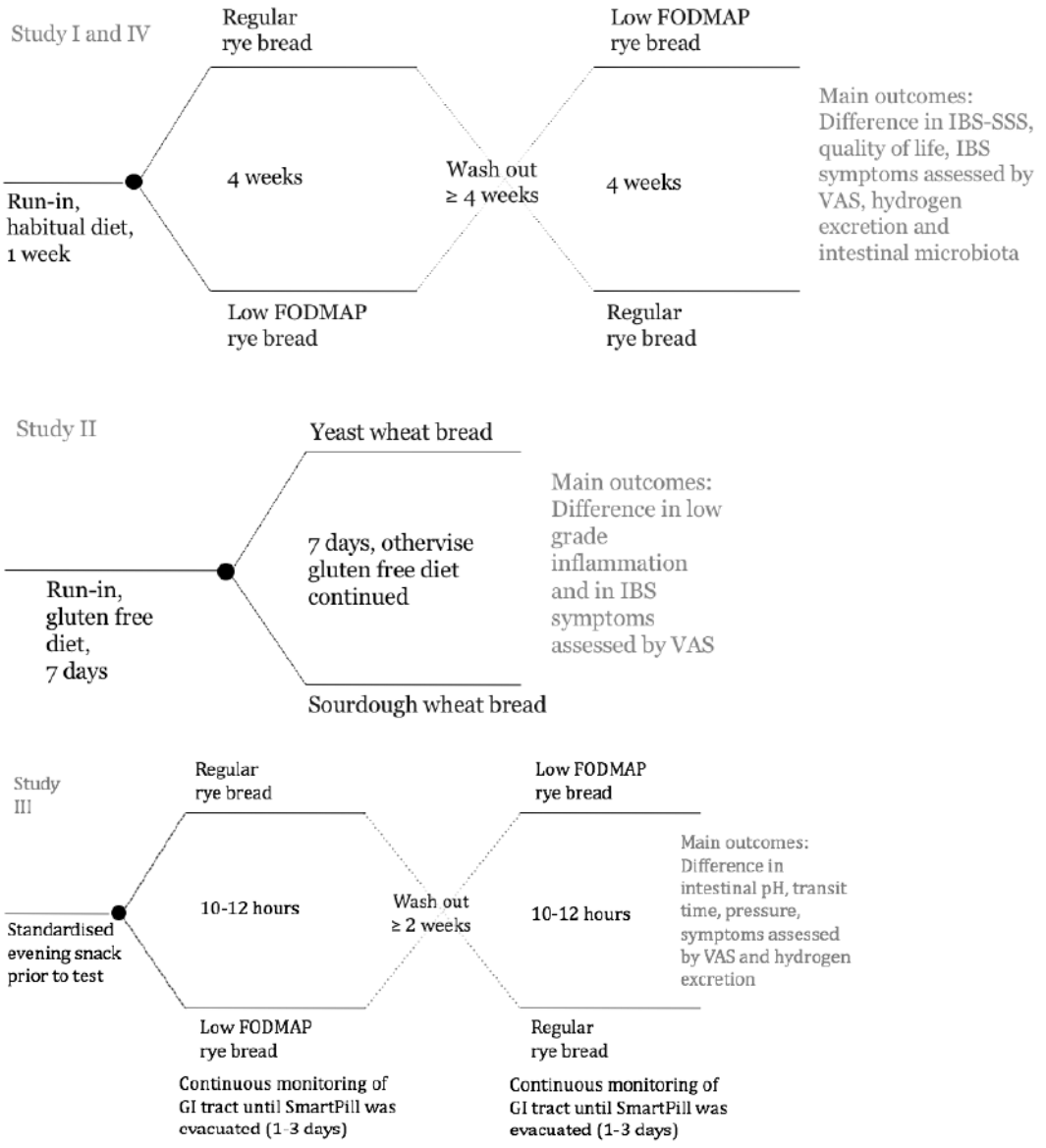


Figure 3. Design of the studies.

In studies I and IV, the exclusion criteria included the presence of an organic GI disease, such as inflammatory bowel disease, coeliac disease, major abdominal surgery, any malignancy, pregnancy or breastfeeding, inability to tolerate rye, a strict low-FODMAP diet or other elimination diet, or taking some medication potentially influencing gastrointestinal function. Study II had otherwise similar exclusion criteria but tolerance to rye was not required. The exclusion criteria were coeliac disease, Crohn's disease, diverticulitis, difficult dyspepsia, stomach bezoar, bowel obstruction, difficult constipation, medication used in the management of intestinal motility, major abdominal surgery, dysphagia, pregnancy or breastfeeding, regular smoking, implanted medical device and hormonal, renal, hepatic or hematologic disease or participation in some other clinical trial during the past two months. The exclusion criteria in study III were otherwise similar as in studies I and IV, except that mild/moderate constipation was allowed; neither attendance in clinical studies during the past two months nor having any medical instrument within the body was allowed. No participants were included in study IV that had not participated in study I, in other words, study IV was a sub-study of study I.

4.2 Dietary intake (I-IV)

Food diaries for 4 days were filled in by the participants in studies I and IV. In study III, 3 day food diaries were used to calculate dietary intake. In study IV, all the food was provided for the participants on the evening before the test and during the test day. Dietary intake was calculated by using a nutrition programme (Aivodiet Ltd, Turku, Finland) in all other studies, except in study III in which the Fineli internet interface (www.fineli.fi) was used to calculate the nutritional composition of the test meals. In studies I and IV, the participants were asked to adhere to their normal background diet whereas in study III, all participants were instructed to follow a strict gluten free diet for the whole study period of 14 days (and breads were provided as the sole source of gluten/wheat).

4.3 Baking of study breads (I-IV)

The breads were developed and supplied by Fazer Bakeries (Vantaa, Finland). Both rye bread recipes (studies I, III and IV) contained the same amount of wholegrain rye flour, wheat flour and other ingredients. The breads had a similar appearance, taste and were packaged in transparent plastic pouches in studies I and IV. The control bread (regular rye bread) was prepared using traditional rye sourdough whereas the low FODMAP rye bread was prepared using a specific sourdough system which resulted in a rye bread with a clearly lower FODMAP content; the fructan content of the low FODMAP rye bread was 0.3 g/100 g whereas that of the regular rye bread was 1.1 g/100 g with the corresponding mannitol contents being 0.1 g/100 g and 0.3 g/100 g, respectively.

The wheat breads (study II) were also developed by Fazer Bakeries. Both test breads were made using the same baker's wheat flour. The industrial yeast wheat bread was prepared using a straight-dough bread making process that lasts for approximately 2 hours (including dough mixing, dough rest, moulding, proofing, oven baking). The recipe of the

yeast wheat bread contained added wheat gluten 2% (of flour weight), bread improvers (such as an emulsifier) and sorbic acid (E200) as a preservative. The sourdough wheat bread was baked using a dough mixer and moulded by hand. The sourdough bread utilised wheat sourdough in the recipe with a long bulk fermentation stage. Overall, the sourdough stage lasted more than 12 hours. No improvers or any added gluten or preservatives were used in the sourdough bread. Both breads were packaged in white plastic pouches in order to prevent the participants from comparing the appearance of the breads.

4.4 FODMAP, ATI and nutritional composition of breads (I-IV)

The nutritional composition of the breads in all studies was analysed by Eurofins scientific Finland, Raisio (Food & Agro), Finland. The dietary fiber content of the breads was determined with the AOAC method 2011.25, discriminating between soluble vs. insoluble, low vs. high molecular weight dietary fibres. The mannitol content was analysed by the HPLC method used by Eurofins Food &Agro, Lidköping, Sweden. The fructan content was analysed by the AOAC 999.03 method (Megazyme assay kit K-FRUC, Megazyme International Ireland Ltd, Bray, Ireland). The nutritional composition along with the FODMAP and ATI contents of the study breads is illustrated in tables 6 and 7.

Table 6. Nutritional composition of the breads examined in studies I, III and IV

	Study I and IV		Study III	
	Low FODMAP rye bread /100 g	Regular rye bread /100 g	Low FODMAP rye bread /100 g	Regular rye bread /100 g
Energy, kJ (kcal)	1024 (245)	1033 (247)	1031 (245)	1037 (246)
Protein, g	9.2	9.3	7.5	7.5
Fat, g	1.3	1.2	2.6	1.1
Carbohydrates, g	43.6	44.0	42.4	45.1
Dietary fibre, g	10.2	10.5	10.8	12.8
Fructans, g	0.3	1.1	0.4	1.2.
Mannitol, g	0.1	0.3	0.1	0.4
Resistant starch	0.9	0.8	Not available	Not available

Table 7. Nutritional composition of the breads examined in study II.

	Sourdough Bread /100 g	Yeast-Leavened Bread /100 g
Energy, kJ (kcal)	972 (229)	1116 (264)
Protein, g	9.3	9.9
Fat, g	1.2	2.4
Carbohydrates, g	43.4	48.4
Dietary fibre, g	3.9	4.5
Fructans, g	0.06	0.23
Resistant starch	0.7	0.8
ATIs*	Monomeric	Dimeric
*) Could not be quantified precisely as methods for quantification of ATIs were not available to the researchers at the time of the study execution.		

The analysis of monomeric, dimeric and tetrameric ATIs was performed by Dr. Xin Huang and Dr. Tuula Sontag-Strohm in the University of Helsinki, Department of Food Science. Sourdough bread and yeast-fermented bread were cut into small pieces, excluding the crusts. Albumin was extracted from 1 g of bread and flour samples into 20 ml buffer containing 10 mM Tris-HCl, 0.1 M EDTA, pH 7.5 at 4 °C for 1 hour with gentle shaking. Following centrifugation at 10000 × g at 4 °C, the supernatant was collected and precipitated with 50% ammonium sulfate at room temperature. The precipitates were dissolved in the same buffer and its protein concentration was determined with a DC protein assay Kit (5000111, Bio-Rad Laboratories Inc, CA, USA) with a bovine serum albumin standard. The protein concentrations of albumin in sourdough bread, yeast-fermented bread and flour were adjusted to the same level. The albumin composition was analysed by SDS-PAGE with 12% Bis-Tris protein gel (NP0341BOX, ThermoFisher Scientific, MA, USA). The albumin solutions were mixed with LDS sample buffer (NP0008, ThermoFisher Scientific), with or without sample reducing agent (NP0009, ThermoFisher Scientific), and then boiled for 3 min. The running buffer used was MOPS (NP0001), and protein standard respectively SeeBlue Plus2 (LC5925, ThermoFisher Scientific). The applied voltage was 200 V, and the run time was 50 min. The gel was stained with Coomassie R-250 brilliant blue.

The baking methods used in the studies resulted in a lower FODMAP content in the low FODMAP rye breads and sourdough wheat bread. The ATI content was measured only in study III, i.e. in the case of wheat breads and wheat flour, and it was found to be reduced in the following order: wheat flour>yeast bread>sourdough bread. With the available methods, we could not accurately quantify the amount of ATIs but instead could demonstrate a reduction/hydrolysis of ATIs in the Western blot analysis (Figure 4).

The mannitol content of rye breads was measured, as mannitol is known to be formed

in rye breads fermented with a sourdough system. The mannitol content of regular rye breads was 0.30 h/100 g in studies I/IV; in study III, it was 0.26 g/100 g. The corresponding values for low FODMAP rye breads were 0.10 and 0.09 g/100 g. The fructan content of regular rye breads was 1.10 g/100 g in studies I/IV; in study III it was 1.2 g/100 g. The corresponding figures for low FODMAP rye breads were 0.30 and 0.4 g/100 g.

The analysis of ATI content was based on the Western blot method. The Western blot image (Figure 4) demonstrates the reduction of tetrameric ATIs of wheat flour; tetrameric ATIs have a molecular weight of approximately 49 000- 62 000 Daltons. In the yeast wheat bread, the tetrameric ATIs are reduced to dimeric ATIs, with molecular weights of approximately 17 000-24 000 Daltons; in the sourdough wheat bread, ATIs are further reduced to monomeric ATIs which have molecular weights in a range 6 000-14 000 Daltons.

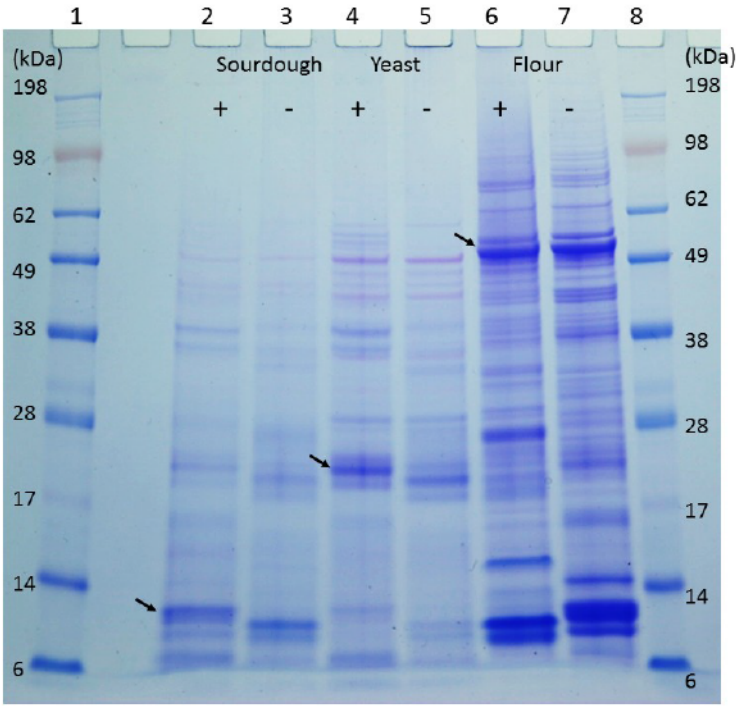


Figure 4. SDS-PAGE of albumins from bread and flour. Lanes 1 and 8, protein molecular weight standard; lanes 2 and 3, albumin extract from sourdough bread; lanes 4 and 5, albumin extract from yeast-fermented bread; lanes 6 and 7, albumin extract from wheat flour. Lanes 2, 4 and 6, marked '+', samples were reduced with dithiothreitol, and those marked '-' were without reduction. Arrows indicate ATIs in different forms, monomer in lane 2, dimer in lane 4 and tetramer in lane 6. Printed with Creative Commons with permission provided by the publisher, MDPI (Laatikainen et al. 2017).

4.5 Adherence to treatments (I-IV)

Adherence to the treatments was monitored in studies I, III and IV by food diaries and special questionnaires designed to evaluate the number of bread slices consumed by each participant. Withdrawal reasons were asked either in person or via email when a participant dropped out from the study. All food was prepared and provided by study assistants in study II and the study nutritionist monitored the consumption of the food.

4.6 Symptoms (IBS-SSS and VAS) and quality of life

Two methods were used for measuring symptoms. In study I, the primary outcome measure was IBS-SSS which is a total score of five different symptoms (Francis et al. 1997). IBS-SSS consists of two questions on abdominal pain, one question on bloating, one on overall satisfaction of bowel habit and one on how much IBS affects/interferes with one's life in general. IBS-SSS was measured twice during each treatment period: after 10 days and at the end of the 4th week.

First, visual analog scale (VAS) which is a subjective measure severity of different symptoms with a scale of 0-100 mm, was used in all studies. the value of 0 mm represents the absence of symptom and 100 mm designates the utmost severity of symptoms. VAS measurements were of primary interest in studies II and III even if they were not used as a basis for the power calculation, i.e. they were not defined as official primary outcomes in any of the studies. VAS is a commonly used method in IBS studies (Shepherd et al. 2008, Biesiekierski et al. 2013, Halmos et al. 2014, Elli et al. 2016) even although it has not been formally validated for overall symptoms control in IBS. The VAS assessmens were done once a week in each treatment week in study I and IV, once a day in study II and hourly in study III.

The quality of life was measured in study I with a special questionnaire developed specifically for the purpose of studying IBS (Drossmann et al. 2000). The quality of life measurements were done once at the end of each period.

The participants were instructed on how to use the different questionaires before starting the treatments.

4.7 Hydrogen excretion –a marker of fermentation (I, III, IV)

Studies I and IV
Hydrogen excretion, a marker of colonic fermentation and intestinal gas production (Rumessen 1992) was measured with a breath hydrogen monitoring device (Gastrolyzer, Bedfont Scientific Ltd, Kent, UK) at the baseline and 12 times at 30 min intervals during the 6 h after eating the study breads. To minimise variables that might affect breath hydrogen production, participants were asked to restrain from vigorous physical activity and instructed to consume a standard breakfast containing three slices of the study breads,

spread, cheese or ham, tomato and/or cucumber and water. Coffee and tea were allowed. Subjects were asked to refrain from eating anything more for the next 6 h after the beginning of the test, i.e. at lunch-time, and to eat a similar lunch, if any, during the two study periods. These tests were performed in the homes of the participants after they had been trained to use the device by the study dietician.

Study III
Breath hydrogen was analysed with the Gastrolyzer® before the test breakfast (0 min) and every 30 minutes for 11 hours (660 min) on the test day and every 3 hours on the following days.

4.8 Gastrointestinal transit time, pH and pressure (III)

An indigestible wireless motility device (SmartPill®, Given Imaging ltd, Yoqneam, Israel) was used in study III. This device contains sensors for temperature, pH and pressure; it is a capsule with the following dimensions; 26.8 mm length and 11.7 mm diameter. It sends data to a receiver device worn by the subject. After the measurement, the data is uploaded to a computer from the receiver and analysed by the MotiliGI® program. The program calculates mean pressure, median pH, contractions/min and transit times based on the changes in pH and temperature, in the different parts of the GI tract. In study III, the capsules were swallowed with the test breakfast and the subjects were requested not to eat for six hours after the meal so that the capsule would proceed to the small intestine. The study period for each subject ended when the capsule was defecated.

4.9 Low grade inflammation (II)

Inflammatory biomarkers interleukin 8 (IL-8), interleukin 6 (IL-6) and lipopolysaccharide binding protein (LBP) were measured at the baseline and after the treatment period with the study breads. Serum samples were taken at Aava Medical Centre laboratorium (Helsinki, Finland) by laboratory technicians and frozen at -22°C until analysed by a biochemist (Hanne Salmenkari) at University of Helsinki, Faculty of Medicine, Department of Pharmacology. Serum IL-6 (BMS213HS, ThermoFisher Scientific, Waltham, MA USA), IL-8 (HS800, R&D Systems, Abingdon, UK) and LBP (HK315-01, LBP; Hycult Biotech, Uden, The Netherlands) were quantified using sensitive enzyme-linked immunosorbent assays (ELISA).

4.10 Measurement of intestinal microbiota (IV)

Fecal samples were collected into tubes by the participants at home at the following time points: baseline and on the 2nd last day of each intervention arm. The participants put the fecal samples immediately into a refrigerator (−20°C) and transported them later to the study centre for DNA extraction which was done within 6 months. Bacterial DNA was extracted from about 125 mg of fecal matter using the Repeated Bead Beating (RBB)

method with modifications for automated DNA purification as described in detail in study IV.

Sample preparation for Illumina MiSeq paired-end sequencing of the hypervariable V3-V4 regions of the 16S rRNA gene was done. Samples with less than reads 3000 were excluded.

4.11 Statistical methods (I-IV)

Study I
The overall IBS-SSS score was the primary outcome variable in the first study. The weekly IBS symptoms, the weekly symptom scores and the IBS-QOL score were chosen as the secondary outcomes in the first study. The sample size calculation for the study was based on the primary variable IBS-SSS. Suitable previously published studies were not available to be utilised in the calculations. Thus, we assumed that the difference between study breads would be at least 50 points on the 500-point IBS-SSS score and that the standard deviation of that difference would be 150 points. In that case, a sample size of 73 would have 80% power to detect a 50 points' difference when using a paired t-test with a 0.05 two-sided significance level. We anticipated that the drop-out rate would be 15–20% and therefore 84–88 patients were targeted for this cross-over study. The patient characteristics were expressed as mean (range) for continuous variables and as number of patients (%) for categorical variables. The primary and the secondary outcome variables were analysed using the repeated measures ANOVA for a cross-over design. The effects of treatment (low-FODMAP rye bread vs. regular rye bread), the time effect and the carry-over effect were also calculated by using repeated measures ANOVA for cross-over design. This is a multivariable parametric method analysing all those effects simultaneously. Differences between study breads were expressed as mean (95% CI).

Area under curve estimates of breath hydrogen test were determined using the absolute breath hydrogen values (AUC 0–360 min). The trapezium rule was used in AUC calculations. The number of slices of study breads was assessed at week 1 and during weeks 2–4. The Wilcoxon signed-rank test was used with respect to the breath hydrogen level and the number of consumed slices of study breads and paired samples t-test was used to compare weight between the low-FODMAP rye bread vs. regular rye bread.

Study II
We performed no sample size calculations because of the pilot-nature of the study and because no clinical benchmark studies existed on ATIs at the time of the initiation of the study. However, the total score for the 12 gastrointestinal symptoms was the primary outcome variable. The patient characteristics are expressed as median (range) for continuous variables and as number of patients (%) for categorical variables.

Dietary intake was measured during the run-in period and during the time when the participants consumed the study breads. Mann-Whitney U test was used to compare the study breads with respect to dietary intake. The quantity of consumed slices of study breads was assessed every day during the 14 -day treatment period. Mann-Whitney U test

was used to compare the average number of consumed slices of study breads. Dietary intake was expressed as median (inter-quartile range) because of the skewed distributions. A p-value ≤ 0.05 was considered statistically significant. The statistical analysis was performed using IBM SPSS Statistics for Windows (versions 22.0 and 23.0, Armonk, NY, USA, IBM Corp).

With regards to the inflammatory markers, the study groups were compared using the analysis of covariance (ANCOVA), where the baseline was included as a covariate. The distributions of IL-8 and IL-6 were skewed to the right and were thus logarithmically transformed before analysis. Due to logarithmic transformation, the comparisons for IL-8 and IL-6 are given as ratios such as yeast wheat bread/sourdough wheat bread, i.e. as proportional differences of logarithmically transformed means. Difference in logarithmic means as such would not be interpretable and therefore a ratio is preferable in comparing logarithmically changed means (Bland and Altman 1996).

Gastrointestinal symptoms and the other symptoms were measured daily using VAS (0-100 mm) during the 7-day run-in period and during the 7-day treatment period with study breads. The run-in period was considered as the baseline. The symptom scores for all symptoms were calculated for baseline and for the treatment period. In addition, the total symptom scores were calculated for gastrointestinal symptoms (mean of 12 symptoms) and for other symptoms (mean of 5 symptoms). Analysis of covariance (ANCOVA) was performed when the symptom scores were compared between the study breads and the symptom score during the baseline was included as a continuous covariate. The differences between study breads (yeast wheat bread vs. sourdough wheat bread) are given as baseline-adjusted means with 95% confidence intervals.

Study III

The breath hydrogen was the primary outcome variable used in the study power calculations. Suitable previously published data was not available, and thus the study power was calculated based on a preliminary test made in Fazer Bakeries in which healthy participants ate regular rye bread or low-FODMAP rye bread and the breath hydrogen content was analysed during a 6 hour postprandial period. The difference in breath hydrogen content (ppm) between fasting and 6 hours after the meal was used to evaluate the number of subjects in the current study. Based on the power calculation, the sample size of 8 would have 80 % power to detect a 25 ppm difference in breath hydrogen when using a paired t-test with a two-sided significance level of 0.05.

The subject characteristics and outcome variables are expressed as a median (range) and as the number of cases for categorical variables. The difference in outcome variables between study periods was calculated using the Wilcoxon signed rank test for related samples. Correlations between mean symptom severity and mean breath hydrogen, and between mean symptoms and SmartPill® indices during the colonic phase, were analysed using the Spearman's rho. Statistical analysis was performed with IBM SPSS Statistics (Version 23, IBM Co., New York, USA) and Microsoft Office Excel 2013 (Microsoft Co., Washington, USA).

Study IV

As this was a sub-study of study I, no official primary endpoint was defined but differences between the treatments in microbiota were of primary interest. Two models were fitted for each taxon: one with all data points and one with only the data points with non-zero values. The effect of variable read count was controlled by using the read count as an offset in all statistical models. Age, BMI and treatment order were used as confounders in all models and subject as a random factor. All p-values obtained from these models were adjusted for multiple testing using Benjamini-Hochberg method, reported as P.adjust and values <0.05 considered as statistically significant. In addition, the spread of the data was checked manually for all significant taxa obtained from the models to avoid reporting false positives. The β-diversity was estimated using Bray-Curtis dissimilarity as the distance measure. The function "CorrelationMap" of the mare package was used to study the associations between bacteria and IBS symptoms. In the univariate data, statistical differences were evaluated using t-test for two groups, and with ANOVA in combination with Tukey's post-hoc test. The microbiota differences between the study groups were analysed using generalised linear models with negative binomial distribution for bacterial genera detected in >10% of the samples.

The patients were also grouped based on the change in their IBS-SSS and abdominal pain scores during the rye bread interventions. The classification of responders was as follows: Either a reduction of IBS-SSS score of at least 50 points, as previously determined by Francis et al. (1997), and/or symptoms of pain more than 10 mm in the VAS (mean of weekly measures) compared to baseline during the low-FODMAP bread, or an increase of IBS-SSS score of at least 50 points and/or symptoms of pain more than 10 mm in the VAS (mean of weekly measures) compared to baseline during the regular rye bread. The symptom data were missing from one patient for each bread period. Therefore, 49 subjects were left for the responder analysis.

The intestinal microbiota analysis of 50 subjects who provided fecal samples and whose samples were analysed included 10 554 - 66 363 (mean 35 757) high-quality MiSeq sequences per sample, representing 357 operational taxonomic units (OTUs) and 84 bacterial genera.

4.12 Ethics (I-IV)

All participants in each study signed the informed consent form prior to the start of the studies and they were free to withdraw at any stage. Participants had an opportunity to contact the study physician or dieticians at any stage in any need. The study protocols were approved by the Ethics Committee of the Hospital district of Helsinki and Uusimaa, Finland. Studies I, II and IV were registered at clinicaltrials.gov and study III was registered at isrctn.com.

5 RESULTS

5.1 Subjects

The baseline characteristics of the participants are presented in Table 8.

Table 8. Baseline characteristics of the participants.

Study (N)	IBS sub-type (%)	BMI, mean (range)	Females (%)	Age, mean (range)	Use of rye/ wheat bread at baseline (%)	Use of any bread at baseline, slices/day, mean (range)
I (N=87)	IBS-D (32.5%), IBS-M (62.5%), IBS-U (5%)	23.4 (17.3–36.6)	87.5%	43 (21–64)	62.5% (rye)	2.9 (0.0-8.0)
II (N=26)	All sub-types of IBS	24.8 (18.0-36.6)	96.2%	42 (21-64)	15.5% (wheat)	3.1 (1.0-7.5)
III (N=7)	All sub-types of IBS	26.4 (19.5.-30.4)	100%	39 (29-51)	N/I	N/I
IV (N=50)	IBS-D (34.5%), IBS-M (60.0%), IBS-U (5.5%)	24.8 (18.0-36.6)	92.7%	44 (21-64)	54.5% (rye)	2.8 (0.0-8.0)

N/I: No information, was not asked

At baseline in study I, the mean of IBS-SSS score was 228 (range 80-430), i.e. these participants suffered on average from moderate IBS according to the classification of Drossman et al. (2011).

The presence of lactose intolerance was reported by 28.7% of the participants entering study I, and by 19.2% of those entering study II. The majority, 69.2%, of the participants in study I had been given a diagnosis of IBS prior to the commencement of the study. The corresponding figure in study II was 77%. Altogether, 78 % reported regular use of some dietary supplement in study I and 92% in study II. Furthermore, 30% of participants took fibre supplements regularly at the baseline of study I and 39% in study II/IV. In study III, these data were not collected as it was a tightly controlled 24-hour acute meal study (no fibre supplements were allowed).

5.2 Dietary intake (I, II and IV)

In study I, fibre intake increased from 21 g/d during the baseline to 27 g/d during the period with low FODMAP rye bread ($P<0.001$) and to 29 g/d during the period with regular rye bread ($P<0.001$). No other statistically significant changes were observed in

study I. No statistically significant changes in the intakes of macronutrients or fibre were observed in study II, neither between the bread periods nor when compared with baseline. Fibre intake increased by 7 grams/day during the low-FODMAP rye bread period and by 8 grams/day during the regular rye bread period as compared to the baseline period ($P<0.001$ for both vs. baseline); no other statistically significant changes were observed between the bread periods in study IV. Dietary intakes were not calculated in study III because it was a tightly controlled feeding study where all food was provided and the only difference between the groups was in the quality of rye bread.

5.3 Symptoms (I-IV)

Study I
We found no significant difference in our primary outcome IBS-SSS between the study bread periods. To refresh, IBS-SSS is a composite outcome (scale 0-500) of four different symptoms during the last 10 days and it was measured twice during the treatment periods of 28 days. The estimated mean was 199 (95% CI 179–220) during the low-FODMAP rye bread vs. 207 (187–227) during the regular rye bread. There was no statistical difference between the bread periods as can be seen in Figure 5.

However, our secondary outcome, VAS measurements of individual symptoms showed statistically significant differences between the bread periods based on the mean values of weekly measurements of symptoms (Table 9). Flatulence, abdominal pain, cramps and stomach rumbling were milder when the subjects were eating the low-FODMAP rye bread (P-values: 0.04; 0.049; 0.01 and 0.001 respectively). There was also a significant difference between the bread periods in the total symptom score, i.e. in the mean of all 10 VAS-based symptoms, 30 (95% CI 27–30) during the low-FODMAP rye bread vs. 33 (95% CI 30–37) when consuming the regular rye bread. The mean difference was –3 (–6 to –1), P = 0.02) favoring the low FODMAP rye bread period.

A carryover effect was observed with respect to cramps but not in any of the other symptoms. A time effect was observed in dyspepsia. Therefore, the results for cramps and dyspepsia were reanalysed using the first treatment period only (ANCOVA). The results based on the first treatment period confirmed the results of the cross-over analysis.

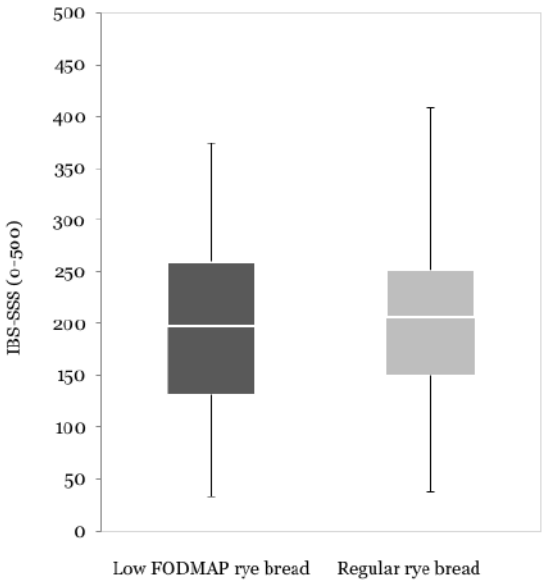


Figure 5. Box-plot on IBS-SSS scores during the treatments period in study I. Whiskers (error bars) above and below the boxes indicate the minimum and maximum values. n=73.

Study II

Gastrointestinal symptoms were not statistically significantly different between the breads when the weekly means were analysed (Table 10). Unexpectedly, tiredness, joint symptoms and decreased alertness were more intense when the subjects were consuming the sourdough bread compared to wheat yeast bread (Table 11, p-values: 0.01; 0.03 and 0.003 respectively). There was no significant difference between the bread treatments in the total gastrointestinal symptom score, i.e. the mean of 12 symptoms which was 27 (SD 12) mm for the sourdough bread vs. 23 (SD 11) mm for the yeast bread. The baseline-adjusted difference between breads was not statistically significant. There was a significant difference between the bread treatments in the non-gastrointestinal symptoms, the mean of 5 symptoms was 26 (SD 18) mm for the sourdough bread vs. 11 (SD 10) mm for the yeast bread. The baseline-adjusted difference in non-gastrointestinal symptoms between bread treatments was 8 (95% CI 2 to 14) mm, p=0.02.

Study III

The mean of the individual gastrointestinal symptoms during the follow-up (30 to 630 min) did not reveal any statistically significant differences between the bread periods (Table 12). The occurrence of flatulence was nearly significantly less after the ingestion of the low-FODMAP rye bread (p=0.06). The difference in AUCs of total symptom score (23 520 (6 885–113 610) mm•min 41 130 (10 785–83 220) mm•min, for low-FODMAP and regular rye breads, respectively) was not statistically significant (p=0.866). No difference

between the bread periods was found in pH, intraluminal pressure or transit times in relation to the symptoms in any parts of gastrointestinal tract.

The correlation between colonic pressure and overall symptom severity during the time when the device was in the colon was significant after the participants had consumed regular rye bread (ρ=0.786, P=.036) and was nearly significant after the low-FODMAP bread consumption (ρ=0.750, P=.052). The correlation coefficients between symptom severity score and colonic contraction frequency were 0.775 (P=.041) and 0.786 (P=.036) respectively.

Study IV
Correlation analysis between the microbiota and hydrogen production or symptoms during the intervention demonstrated two significant associations; hydrogen excretion was positively correlated with the abundance of Anaerostipes (r=0.31, p= 0.003), and a weak positive correlation was observed between constipation and Clostridia FamilyXIII IncertaeSedis (r=0.17, P= 0.035).

A responders' analysis was conducted on the patients who experienced the strongest relief in symptoms during the interventions; this examined if patients who gained symptom relief (responders) after consumption of the low-FODMAP bread had a different microbiota composition as compared to the others (non-responders).

Based on the criteria described in the methods, we identified 23 responders and 26 non-responders. The individual GI symptoms that differed significantly between the groups were flatulence (P=0.004), dyspepsia (P=0.005) and heartburn (P=0.04). They were significantly decreased in the responders during the intake of low-FODMAP bread. The excreted breath hydrogen was significantly higher in the non-responders (P=0.01).

Considering the overall microbiota, a higher fraction of the variation was attributable to the low-FODMAP bread-related responder status (2%, P=0.005) than the intervention itself (1%, P=0.3). The abundance of *Blautia* was higher in the responders at baseline (P.adjust=0.01, fc=1.6) and the abundance of *Barnesiella* (Porphyromonadaceae, Bacteroidales) was lower in the responders at baseline (P.adjust =0.03, fc=0.22). In summary, compositional microbiota differences between the response groups were evident at baseline, but not at the end of the interventions.

We also compared the microbiota between the subjects whose symptoms were strongly triggered after the intake of the regular rye bread to those without a major symptom change. Here, we identified 19 responders and 30 non-responders, with no microbiota differences between the groups (for all taxa P.adjust >0.05).

Table 9. The mean of the VAS scores (0-100 mm) of weeks 1, 2, 3 and 4, Study I (n=73). Printed with Creative Commons permission as provided by the publisher, Wiley (Laatikainen et al. 2016).

Symptom	Low FODMAP rye bread Mean (95% CI)	Regular bread Mean (95% CI)	Difference Mean (95% CI)	P *
Flatulence	47 (43-52)	52 (47-56)	-4 (-8 to -0)	0.04
Diarrhoea	23 (19-27)	25 (21-30)	-2 (-7 to 2)	0.31
Constipation	24 (19-29)	25 (20-30)	-1 (-5 to 3)	0.66
Abdominal pain	34 (29-38)	38 (33-43)	-5 (-9 to -0)	0.049
Cramps	19 (16-23)	25 (20-29)	-6 (-10 to -2)	0.01
Rumbling	33 (28-37)	39 (34-44)	-6 (-10 to -3)	0.001
Heartburn	22 (18-27)	21 (17-25)	2 (-2 to 5)	0.34
Dyspepsia	31 (25-36)	34 (29-40)	-4 (-8 to +0)	0.06
Incomplete defecation	40 (35-46)	41 (36-47)	-1 (-5 to 3)	0.66
Urgency in defecation	29 (25-33)	33 (27-38)	-4 (-8 to 1)	0.11
Total symptom score	30 (27–33)	33 (30–37)	-3 (-6 to -1)	0.02

*) repeated measures ANOVA for cross-over design

Table 10. Gastrointestinal symptoms (VAS 0-100mm) and gastrointestinal total score during the baseline (mean of days 1-7) and during consumption of the study breads (mean of days 8-14), study III. Printed with Creative Commons permission as provided by the publisher, MDPI (Laatikainen et al. 2017).

	Sourdough bread (n=13)		Yeast bread (n=13)		Sourdough bread vs. yeast bread	
Symptom	Baseline	Study bread	Baseline	Study bread	Baseline-adjusted Difference	P value*
Flatulence	25 (16)	37 (20)	22 (12)	32 (17)	3 (-11 to 17)	0.66
Bloating	23 (17)	38 (16)	23 (15)	38 (20)	1 (-13 to 15)	0.93
Diarrhea	10 (8)	13 (15)	12 (10)	13 (10)	1 (-9 to 10)	0.90
Constipation	16 (13)	22 (17)	14 (13)	24 (20)	-4 (-16 to 8)	0.54
Abdominal pain	16 (14)	32 (15)	20 (15)	29 (15)	4 (-7 to 15)	0.44
Abdominal cramps	12 (17)	27 (19)	10 (10)	16 (13)	10 (-1 to 21)	0.06
Borgorygmia	20 (20)	34 (24)	19 (12)	23 (13)	10 (-1 to 21)	0.07
Heartburn	13 (16)	18 (16)	9 (7)	12 (10)	3 (-5 to 12)	0.43
Dyspepsia	14 (14)	29 (17)	18 (17)	24 (23)	8 (-6 to 22)	0.25
Incomplete defecation	30 (25)	37 (25)	24 (16)	32 (24)	-1 (-14 to 13)	0.93
Urgent need for defecation	18 (14)	21 (19)	20 (15)	21 (16)	1 (-10 to 12)	0.90
Nausea	12 (17)	21 (15)	10 (16)	15 (16)	5 (-4 to 13)	0.29
Gastrointestinal total score	17 (12)	27 (12)	17 (9)	23 (11)	4 (-4 to 11)	0.33

Results are given as mean (SD) and mean (95% CI).
*) The study breads were compared using the analysis of covariance (ANCOVA), where the symptom score during the baseline was included as a covariate

Table 11. Non-gastrointestinal symptoms (vas 0-100mm) and their total score during the baseline (mean of days 1-7) and during consumption of the study breads (mean of days 8-14), study II. Printed with Creative Commons permission as provided by the publisher, MDPI (Laatikainen et al. 2017).

	Sourdough bread (n=13)		Yeast bread (n=13)		Sourdough bread vs. Yeast bread	
Symptom	Baseline	Study bread	Baseline	Study bread	Baseline-adjusted Difference	p value*
Tiredness	30 (24)	40 (25)	17 (15)	14 (15)	16 (4 to 27)	0.01
Joint symptoms	17 (19)	23 (21)	9 (14)	7 (13)	7 (1 to 13)	0.03
Skin rash	11 (18)	14 (18)	3,5 (3)	5 (5)	3 (-5 to 11)	0.45
Decreased alertness	22 (25)	35 (26)	12 (15)	13 (12)	14 (5 to 22)	0.003
Loss of appetite	12 (18)	19 (15)	9 (12)	15 (15)	2 (-6 to 9)	0.62
Other symptoms total core	19 (17)	26 (18)	10 (11)	11 (10)	8 (2 to 14)	0.02

Results are given as mean (SD) and mean (95% CI).) The study breads were compared using the analysis of covariance (ANCOVA), where the symptom score during the baseline was included as a covariate.

Table 12. Means (mm) of the gastrointestinal symptoms on VAS during 30-630 minutes after the test meals; median (range), study III. Printed with Creative Commons permission as provided by the publisher, Baishideng Publishing Group (Pirkola et al. 2018). (n=7)

	Low-FODMAP rye bread	Regular rye bread	p-value*
Abdominal pain	2.4 (0.0–28.2)	4.8 (0.1–21.9)	0.74
Cramps	1.2 (0.1–29.2)	3.0 (0.1–10.0)	0.92
Bloating	12.3 (1.0–48.4)	23.1 (1.1–37.8)	0.87
Flatulence	3.3 (0.5–7.6)	4.2 (0.5–28.2)	0.06
Rumbling	3.0 (0.1–6.6)	3.8 (1.4–11.4)	0.40
Nausea	1.0 (0.0–22.4)	2.1 (0.0–15.5)	1.00
Heartburn	1.4 (1.0–20.8)	1.8 (0.1–20.3)	1.00
Dyspepsia	7.4 (1.0–26.1)	11.8 (1.2–23.2)	0.31
Urge of defecation	1.5 (0.0–29.5)	2.1 (0.3–22.4)	0.74

*Wilcoxon signed rank test

5.4 Quality of life (I)

No significant difference was observed between the study breads in the quality of life. The mean (95% CI) was 29.2 (25.0–33.5) during the low FODMAP rye bread vs. 30.0 (25.5–34.5) during the regular rye bread. The difference was −0.8 (−2.9–1.3), P = 0.45.

5.5 Hydrogen excretion (I, III)

Study I

Breath hydrogen excretion was significantly lower during the low-FODMAP rye bread meal as compared to the regular rye bread meal (Figure 6). The median (inter-quartile range) AUC was 52.9 (32.4–76.8) ppm during low-FODMAP rye bread vs. 72.6 (46.7–114.9) ppm during the regular rye bread (P = 0.01, Wilcoxon signed-rank test). Only subjects for whom there were complete breath hydrogen measurements during both study breads were included into the analysis (n = 60).

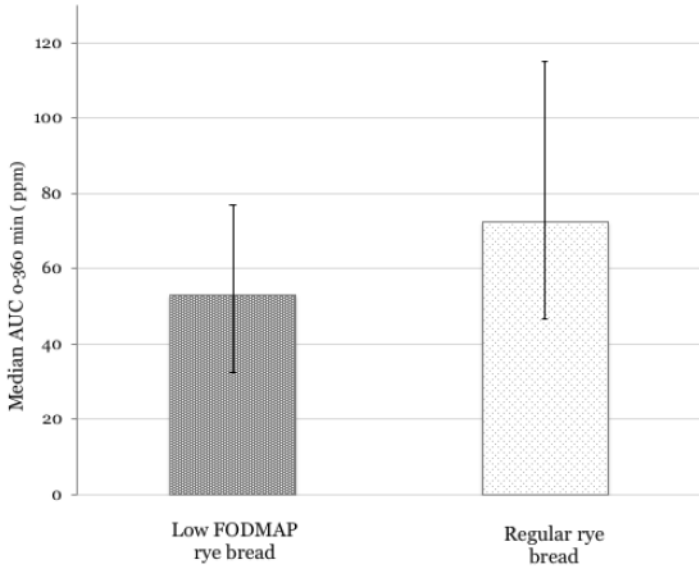


Figure 6. $AUC_{0\text{-}360min}$ of breath hydrogen excretion during the test meals (p=0.01, Wilcoxon signed ranks test). Whiskers (error bars) above and below the columns indicate the inter-quartile ranges. (n=60).

Study III

Postprandial excretion of hydrogen expressed as AUC (0-630 min) was [median (range)] 6300 (1785-10800) ppm•min for low-FODMAP rye bread and 10 635 (4215-13080) ppm•min for regular bread. The two bread tests differed significantly (p=0.028) indicating that there was more intensive colonic fermentation when the participants were consuming the regular rye bread. Figure 7 illustrates the hourly development of hydrogen excretion after the test meals.

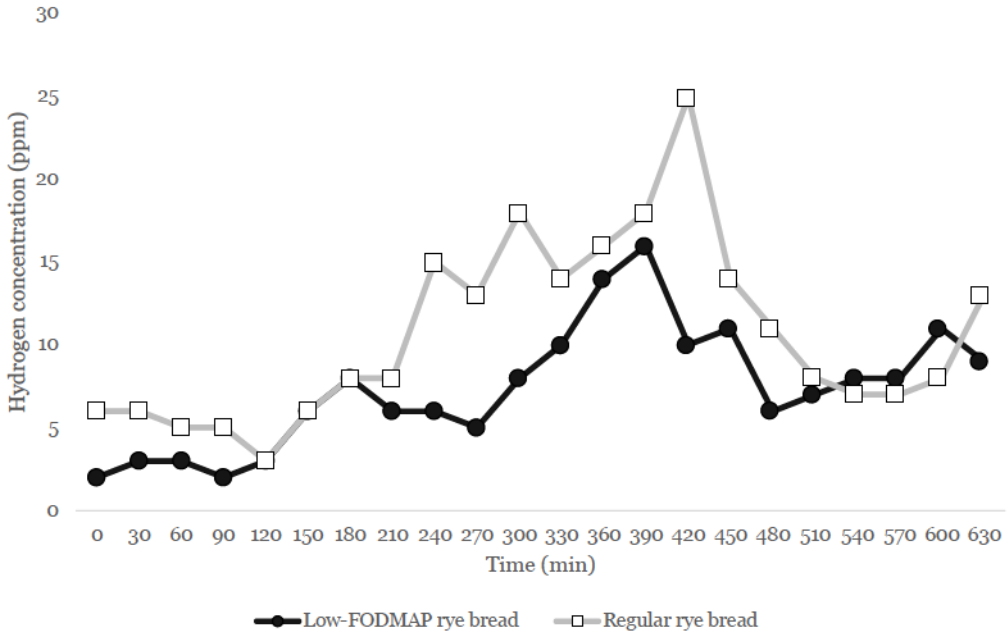


Figure 7. Medians of expired hydrogen concentration (ppm•min) after consumption of the study breads during the test days. Printed with Creative Commons permission as provided by the publisher, Baishideng Publishing Group (Pirkola et al. 2018). (n=7)

5.6 Low grade inflammation (II)

None of the serum inflammation markers differed statistically significantly between the treatments; the baseline-adjusted difference ratio for IL-6 was 0.91 (95% CI 0.73-1.14, P=0.94), for IL-8 it was 1.01 (CI95% 0.74-1.39, P=0.38) and baseline adjusted difference in mean for LBP was -1.19 (95% CI -2.55-0.17, P=0.08) (Figure 8).

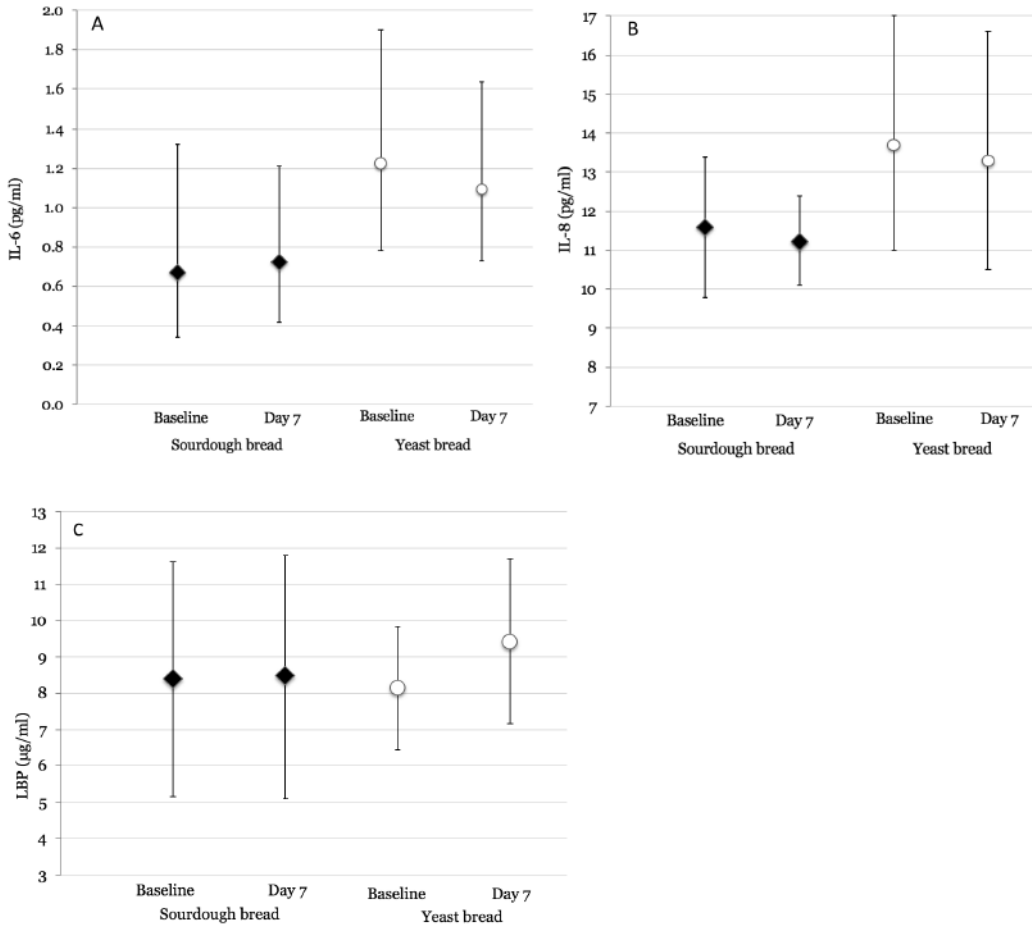


Figure 8A,B,C A) S-IL-6 (pg/ml), B) S-IL-8 (pg/ml) and S-LBP (µg/ml) at the baseline and after the treatment periods (Day 7) with sourdough (◆) and yeast (○) breads. Dashes/circles with error bars indicate geometric means with 95% confidence intervals for IL-8 and IL-6, and arithmetic mean with 95% confidence intervals for the LBP level. (n=24)

5.7 Gastrointestinal transit time, pH and pressure (II)

Transit times, medians of pH-values, mean pressures and contractions can be found in Table 13. These measures from any part of the gastrointestinal tract did not differ between the bread tests. An association was observed between the colonic pressure and the overall symptom severity. The correlation was statistically significant when the participants had been consuming regular rye bread (ρ=0.786, p=0.036) and was nearly significant during low-FODMAP bread consumption (ρ=0.750, p=0.052). The correlation coefficients

between symptom severity and colonic contraction frequency were 0.775 (p=0.041) and 0.786 (p=0.036) after regular and low-FODMAP breads. Colonic pH and hydrogen excretion were associated with symptom severity after the regular bread (ρ=0.821, p=0.023 and ρ=0.857, p=0.014, respectively) but not after low-FODMAP bread (ρ=0.342, p=0.452 and ρ=0.536, p=0.215, respectively).

Table 13. SmartPill derived transit times, pH-values, mean pressure and contractions/min; median (range). Printed with Creative Commons permission provided by the publisher. (n=7)

	Low-FODMAP rye bread	Regular rye bread	p-value*
Transit time (h)			
Stomach	18.1 (5.3–22.3)	5.6 (4.5–18.0)	NS
Small intestine	4.0 (2.1–5.6)	4.6 (3.2–6.6)	NS
Colon	25.2 (12.2–50.0)	32.1 (14.7–47.6)	NS
Whole GI tract	46.5 (22.6–73.5)	45.8 (24.3–70.4)	NS
Median pH			
Stomach	1.5 (0.8–4.1)	1.5 (1.0–2.4)	NS
Small intestine	7.5 (5.0–8.0)	7.6 (7.0–7.8)	NS
Colon	7.2 (5.8–7.5)	6.5 (5.9–8.5)	NS
Mean pressure (mmHg)			
Stomach	2.2 (1.9–2.7)	2.5 (2.0–3.0)	NS
Small intestine	3.1 (1.6–8.6)	4.5 (2.4–7.0)	NS
Colon	4.8 (3.2–6.3)	4.0 (2.0–6.7)	NS
Contractions /min			
Stomach	1.2 (0.6–1.7)	1.0 (0.8–1.8)	NS
Small intestine	3.2 (0.5–6.1)	4.9 (1.9–6.5)	NS
Colon	1.7 (1.3–2.9)	1.7 (0.6–3.3)	NS

*Wilcoxon signed rank test

5.8 Microbiota (IV)

The microbiota of the subjects at baseline consisted of *Actinobacteria* (mean 5.3 %), *Bacteroidetes* (7.8 %), *Firmicutes* (86.3 %), *Proteobacteria* (0.3 %) and *Verrucomicrobia* (0.3 %). The most abundant bacteria at the genus level were *Lachnospiraceae IncertaeSedis* (mean relative abundance 20.1%), *Bacteroides* (12.6%), *Faecalibacterium* (9.9%), *Blautia* (9.0 %) and *Subdoligranulum* (7.8%).

Principal coordinates analysis (PCoA) was performed in order to estimate β-diversity (community dissimilarity), using Bray-Curtis dissimilarity as the distance measure. There was no distinct microbiota clustering nor significant separation based on the treatments or timepoints (P>0.05). Permutational multivariate ANOVA, with Bray-Curtis dissimilarities, was used to assess the proportion of variation in the microbiota composition attributable to the time points (baseline, 1st and 2nd bread) and the treatments (low FODMAP rye bread and regular rye bread). Only minor effects on the overall community structure were observed as the time points and treatment order both explained only 1% of the microbial variation in these patients (P>0.5). In addition, neither α-diversity nor richness was affected by the intervention (P>0.4 for all comparisons).

When comparing the relative abundance of bacterial genera between the two interventions (Figure 9), the abundance of *Klebsiella* was lower when the subjects were consuming the low FODMAP rye bread compared to the time when they ate the regular rye bread (P.adjust=0.048, fc= 14.8). This was the only statistical difference in bacterial taxa when comparing the breads. Both breads induced modest, partly overlapping effects on the microbiota. Of all the analysed bacterial taxa, five differed between the low FODMAP rye bread and baseline (Figure 9). During the low FODMAP rye bread period, the abundance of *Bacteroides* (P.adjust=0.03, fold change fc= 1.59), two Firmicute genera i.e. *Flavonifractor* (Ruminococcaceae, P.adjust =0.06, fc=1.55) and *Holdemania* (Erysipelotrichaceae, P.adjust=0.08, fc=1.84), and two Proteobacterial genera i.e. *Klebsiella* (Gammaproteobacteria, P.adjust=0.05, fc=136.14) and P*arasutterella* (Betaproteobacteria, P.adjust=0.04, fc=1.97) were reduced in comparison to baseline.

The only difference between the regular rye bread versus baseline samples was the reduction in *Flavonifractor* (Ruminococcaceae) (P.adjust=0.01, fc=14.83). There was a trend towards an increase in the abundance of Bifidobacterium during the low-FODMAP rye bread period (P=0.03, P.adjust=0.22, fc=1.42), and a similar but weaker upward trend was observed during the regular rye bread period (P=0.28, P.adjust=0.70, fc=1.20). The interventions had no effects on other bacteria including *Faecalibacterium prausnitzii*, *Akkermansia muciniphila* and Lactobacillus spp.

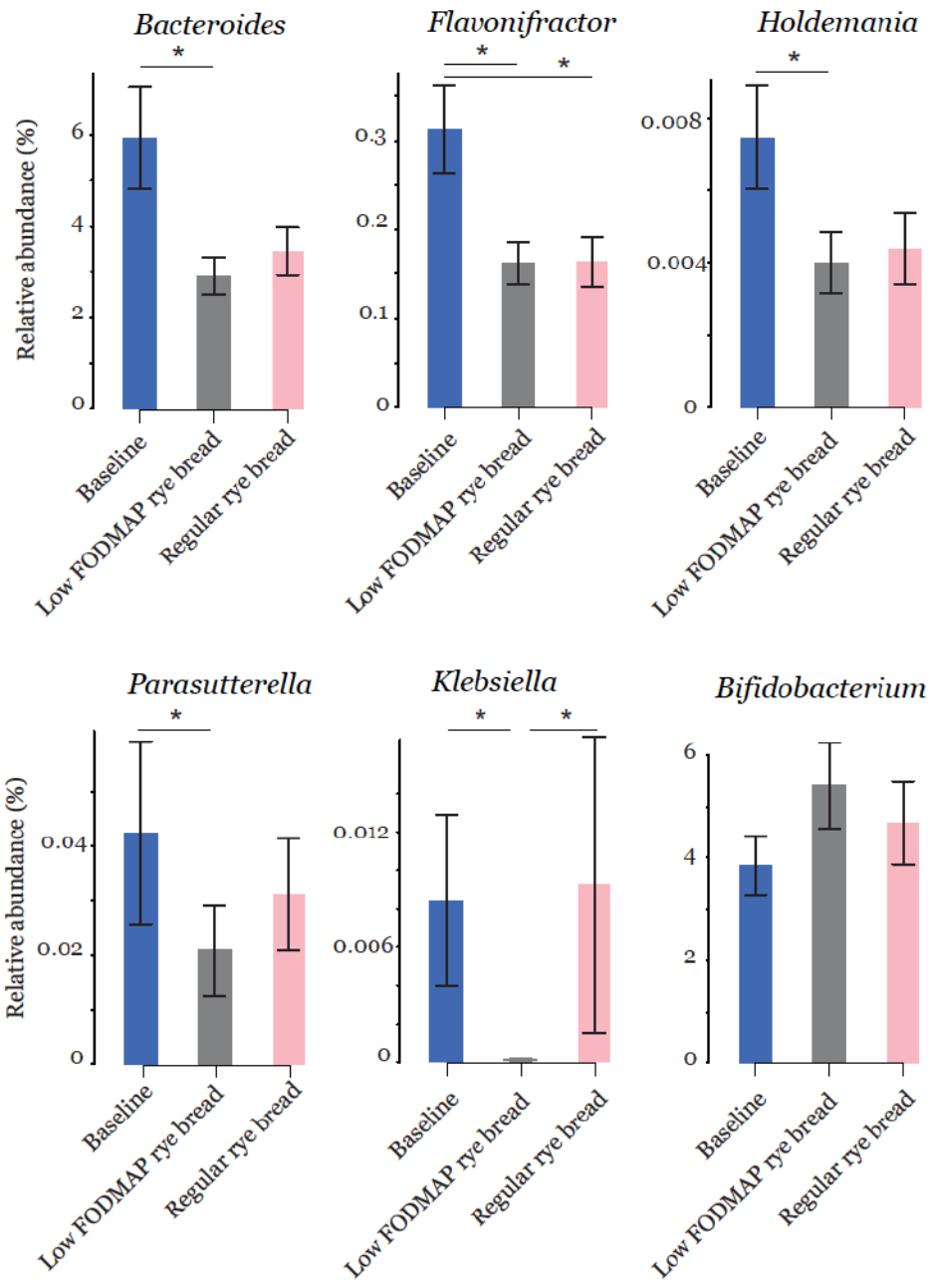


Figure 9. Relative abundance (± standard error) of the bacterial genera that were significantly different between the bread treatments. Significant differences calculated with negative binomial models in mare R package are indicated by an asterisk (adjusted $p<0.05$).

6 DISCUSSION

6.1 General discussion

Hydrogen excretion and symptoms
Studies I and III demonstrated that the low FODMAP rye bread reduced gas formation measured by the hydrogen excretion test when compared to regular rye bread as hypothesized. No other studies conducted in acute meal tests among healthy subjects have assessed the effects of rye products on gas formation in IBS. Previous studies in healthy subjects have shown that rye products produce more intestinal gas than white wheat bread (Nilsson et al. 2008, Rosén et al. 2011). The formation of gas in the colon seems to be partly dependent on the FODMAP content of the bread.

There are no previous randomised studies on rye products among people with Rome criteria defined IBS. A reduction was observed in flatulence, abdominal rumbling, cramps and pain in the 4-week study I but not in the acute meal study II. The choice of a low-FODMAP bread as the only dietary modification did not seem have a large effect on IBS symptoms because there did not appear to be any difference in IBS-SSS or quality of life which both assess symptom control more holistically than VAS measurements on individual symptoms. Consequently, it is unlikely that simply by consuming a bread low in FODMAPs would help an indivividual alleviate all IBS symptoms, or achieve adequate symptom control.

The observation that rye products high in FODMAPs may provoke some IBS symptoms is supported by a study conducted among healthy individuals (Vuholm et al. 2017); these investigators examined the effects of rye, whole wheat bread and white wheat bread on metabolic health, microbiota and GI symptoms. Their 6-week parallel study demonstrated that rye, whole wheat and white wheat all increased abdominal bloating and fatigue when consumed ad libitum. In addition, the study of Lappi et al. (2014) included healthy patients with mild rye-related GI symptoms into their study. The authors reported that rye bread had increased flatulence when compared to white wheat bread.

Furthermore, Paajanen et al. (2004) have also shown, as confirmed here, that in people with functional gastrointestinal symptoms, rye products with high FODMAP contents cause more abdominal discomfort than white wheat similarly low in FODMAPs. They studied individuals with subjective milk intolerance and exposed them to rye and white wheat products. It was found that people with a subjective milk intolerance without lactose intolerance experienced more symptoms from rye than from wheat; bloating, flatulence and abdominal pain and their total symptom score was higher during the rye bread period vs. white wheat bread period. The most likely explanation for the observed difference is that rye generates more intestinal gas than wheat due to its content of fermentable carbohydrates, i.e. FODMAPs (Nilsson et al. 2008, Ibrügger et al. 2014).

No differences were found in gastrointestinal symptoms between the breads in our wheat bread study (II) despite the lower ATI and FODMAP content in the sourdough bread. One can speculate that the failure to alleviate gastrointestinal symptoms might have been due to a nocebo effect, masking any true differences, or perhaps there was too small a difference in the FODMAP intake achieved by the breads (0,3 g/d). Another explanation is the fact that the study groups were slightly dissimilar at baseline. Moreover, it is also theoretically possible that the ATI reduction achieved in sourdough bread was not sufficiently extensive. No previous clinical studies have compared sourdough and regular wheat bread in subjects with IBS but a 6-week randomised study compared modern (common) wheat products and Kamut (ancient) wheat products in IBS (Sofi et al. 2014). Ancient wheat varieties are known to be lower in FODMAPs and ATIs (Ziegler et al. 2016, Zevallos et al. 2017) but in their study, the authors did not measure either the ATI or the FODMAP contents of regular or Kamut wheat products (Sofi et al. 2014). Nonetheless, Kamut products resulted in improved gastrointestinal tolerance which suggests that FODMAPs and ATIs might play a role in non-coeliac gluten sensitivity. It is noteworthy that in this trial the differential effects of breads were only shown after 4-6 weeks. It is thus possible that seven days, the length of observation in the present study, was too short to allow detection of differences between the treatments.

The sourdough wheat bread used in the present study was lower also in gluten than the yeast bread because gluten was added only into the yeast wheat bread (a regular practice in commercial production). A recent randomised study demonstrated that enzymatic processing of dough in order to reduce gluten content of bread does not help in reducing symptoms in non-coeliac gluten sensitivity (Rees et al. 2018). These results are further evidence that gluten might not be the key factor evoking gastrointestinal symptoms related to grains, albeit that study was also short, as was study II.

Furthermore, the present study with wheat breads does not support the claim that commonly used baking additives such as yeast, added gluten, sorbic acid or emulsifiers would be major drivers of IBS symptoms.

Inflammation, pH, transit time and intraluminal pressure
Despite a substantial reduction of putative pro-inflammatory substances such as ATIs (Junker et al. 2012, Zevallos et al. 2017) in sourdough wheat bread, we could not demonstrate any changes in the systemic markers of inflammation examined in study II; IL-6, IL-8 and LBP remained unchanged also during the yeast bread period of seven days. This finding is in contrast to previous pre-clinical studies (Junker et al. 2012, Zevallos et al. 2017) that have suggested that the ATIs present in wheat cause relatively rapid changes in inflammatory markers. These present findings may be attributed to the design and methods used in our study (see next chapter), or alternatively, our findings suggest that during an otherwise gluten free diet, the ATIs in yeast wheat bread are not capable of inducing clinically relevant inflammation when consumed in moderation (5 slices/day).

It was determined that the SmartPill® stayed in stomach much longer (4.5-22 hours; mean stomach emptying for regular bread period was 5.6 hours and low FODMAP bread period 18.1 hours) than previously reported in most trials, even although a 6 hours' break in

eating was adopted after breakfast to ensure emptying of the stomach. Only one trial has previously reported a slightly lengthened stomach transit time in a subset of IBS-C subjects (Ringel-Kulka et al. 2015), but others have consistently reported substantially shorter transit times in the stomach. Emptying of the SmartPill® capsule occurred at about 4 h in the investigations conducted by Kuo et al. (2007) and Casidilly et al. (2008). Thus, our study implies that the SmartPill might not be an optimal device to evaluate the gastrointestinal function in IBS during meal studies lasting less than 24 hours, due to the device's inability to measure gastric properties in a timely manner. Based on our findings on hydrogen excretion during the rye bread treatments, it is obvious that the rye bolus moved from the stomach to the intestine many hours before the SmartPill®.

There was a correlation between increased intracolonic pressure and symptom severity, and it is argued that this further emphasizes the central role of visceral sensitivity in IBS. This finding hints that IBS symptoms might be worsened by any property that increases the colonic pressure. There do not appear to be any other SmartPill derived outcomes in IBS suggesting outcomes similar to ours or refuting our findings.

No other comparative dietary studies using SmartPill® exist among subjects with IBS; this present study was first of its kind. SmartPill® has been used only in one previous study among subjects with IBS (Ringel-Kulka et al. 2015); the researchers demonstrated that IBS patients have a lower intracolonic pH when compared to healthy subjects, which they interpreted as higher colonic fermentation in these patients but no dietary intervention was performed in that study.

Microbiota

There were only modest changes in microbiota after consumption of the rye breads. There was only one statistically significant difference between the bread periods i.e. in the abundance of *Klebsiella*, their amount was lower during the low FODMAP rye bread period. Previously, overgrowth of *Klebisella* in the small intestine of IBS patients has been documented; a microbial specimen was obtained via endoscopy as aspirates (Giamarellos-Bourboulis et al. 2015). It is possible that consumption of low FODMAP rye bread reduces the abundance of *Klebsiella* in the small intestine but this is speculative as it was not possible to determine the origin of *Klebsiella* in the gastrointestinal tract.

However, some other changes were observed when compared to baseline. Changes in microbiota were more prominent during the low FODMAP rye period than during the regular rye bread. The abundances of *Bacteroides, Flavonifractor, Holdemania, Parasutterella* and *Klebsiella* were reduced during the low FODMAP rye bread period, whereas only the abundance of *Flavonifractor* was reduced during the regular rye bread period. With regards to other microbial factors, no statistically significant changes were observed although there was an increased abundance of bifidobacteria when the particuipants consumed a low FODMAP rye period in the uncorrected model of analysis. Currently, as far as is known, no other studies reporting effects of rye or wheat products on microbiota of IBS subjects have been published.

Some studies comparing either regular rye or whole grains vs. refined grains have reported outcomes on the microbiota of healthy subjects (Lappi et al. 2013, Vuholm et al. 2017, Roager et al. 2017, Vanegas et al. 2017). In these studies, the observed effects on microbiota have also been modest. Lappi et al. (2013) found a 37% decrease of Bacteroidetes in the refined grains group compared to baseline. Furthermore, they also found that there was a decreased abundance of bacteria related to *Bacteroides vulgatus, B. plebeius,* and *Prevotella tannerae*, whereas those of bacteria related to Collinsella and members of the Clostridium clusters IV and XI were increased when refined grains were consumed. Vuholm et al. (2016) did not observe any differences in the abundances of any taxa between any of the diet groups (whole grains, rye or refined grains) in healthy but overweight subjects. Roager et al. (2016) found out that the abundance of *Faecalibacterium prausnitzii* and one *Prevotella copri* increased after the whole grain period but their abundance decreased after the refined grain consumption. In contrast, the abundance of *Bacteroides thetaiotaomicron* changed in the opposite direction but no significant changes were found in the abundance of individual bacterial species. Venegas et al. (2017) detected a significant relative change toward a decrease in *Enterobacteriaceae* abundance in the whole grains group as compared to the refined grains group. At the genera level, there was a significant relative change toward an increase in *Lachnospira* abundance in the whole grains group compared to the refined grains group (Venegas et al. 2017). Taken together, these data show that no uniform change in microbiota is observed when whole wheat or rye is consumed regularly. The clinical significance of our observations on microbiota regarding low FODMAP rye bread remains to be determined.

The only significant finding in regards to macronutrient intake found here was the increased intake of dietary fibre during the rye periods as compared to baseline (study I and IV). The intake of fibre was increased by 6 grams in the 4-week rye study during the low FODMAP rye bread periods and by 9 grams during the regular bread period. This increase in the intake of dietary fibre during the low FODMAP rye bread periods was almost as high as that achieved when using 10 grams of psyllium daily or 20 grams of wheat bran/day which are the typical doses which have been used in randomised IBS trials (Bijkerk et al. 2004). The consumption of a low FODMAP rye bread might be a feasible means of achieving a higher intake of dietary fibre among people with IBS, and potentially in this way, reduce the risk of chronic diseases such as colorectal cancer (Aune et al. 2011) or cardiovascular diseases (Threapleton et al. 2013).

Given that there was a lower than recommended intake of dietary fibre (approximately 21 grams/day) in our patient populations and in some other IBS populations following low FODMAP diet (Böhn et al. 2015, Harvie et al.2017), consumption of a low FODMAP rye bread may help a patient with IBS to reach the recommended intake of fibre. The major fibre component responsible for the increased intake of fibre in low FODMAP rye periods was arabinoxylan which is the dominant component of fibre in rye and barley (Frølich et al. 2013) although the intakes of lignin, cellulose and resistant starch were also increased.

6.2 Methodological considerations

The major strength of this series of studies is that they were double blinded. It is almost impossible to mask a holistic low FODMAP diet from both investigators and study subjects but the present study designs allowed masking of the true nature of treatment and control breads. Thus, the present rye studies and the acute meal study, are an important piece of evidence further strengthening the relevance of the FODMAP concept in IBS i.e. the double blind setting and the cross-over design help to avoid the placebo effect often considered as a challenge in low FODMAP studies (Kroogsgaard et al. 2017).

The reduction of FODMAPs in the developed breads was successful and verified in the laboratory analyses. The FODMAP contents in the low FODMAP rye bread and the sourdough wheat bread were approximately 70% lower than in control breads (Tables 6 and 7), i.e. the regularly consumed counterparts. Our results are in line with other studies highlighting the potency of long proofing of dough to reduce the FODMAP content of bread (Ziegler et al. 2016, Zamaratskaia et al. 2018), and in that way make sourdough breads more suitable for IBS patients. Interestingly, Ziegler et al. (2016) also showed that it does not have to be a sourdough technique but using common baker's yeast also results in a reduced FODMAP content of bread if the proofing time is long enough. It was demonstrated that a proofing of the dough for more than 4.5 hours with yeast reduced the amount of FODMAPs by up to 90%. Based on their results, a reduction of FODMAPs may not be specific to the sourdough method but related to the duration of the proofing period, i.e. a long proofing time is needed to achieve substantial fermentation. Currently, most industrial wheat breads are baked using quick proofing times, i.e. the Chorleywood method, which typically employs less than 1.25 hours' proofing times (Cauvain and Young 2005).

There is no other data available on the effect of sourdough fermentation of wheat on the ATI-levels in bread. In this respect, the present outcomes provide innovative information and question the potential central role of ATIs in wheat sensitivity. No other clinical study has compared a wheat product high in ATIs to a wheat product with reduced ATIs.

The methods used here made it possible to scrutinize the effect of smaller differences in the intake of FODMAPs better than in most previous studies. In addition to the report of Skodje et al. (2017), the present rye studies are some of the first to demonstrate that a difference of 2 grams in the intake of FODMAPs per day increases gas formation and can consequently trigger some gastrointestinal symptoms among patients with IBS or non-coeliac wheat sensitivity. On the other hand, the present wheat study suggested that a difference in the intake of fructans of 0.3 grams per day is unlikely to evoke any discernible effects on GI symptoms. In holistic low FODMAP diet studies, the difference in the intake of FODMAPs has been typically more than 10 grams per day (Ong et al. 2010, Staudacher et al. 2011, Halmos et al. 2014) and in these trials, larger benefits have been achieved in the control of symptoms.

Our studies allowed the participants to consume their habitual diet and did not switch patients to a low FODMAP diet. It can be argued that this approach is more valid from the long-term perspective than having low FODMAP diet as a background diet. Adherence to a strict low FODMAP diet is only intented for short periods of time (Whelan et al. 2018), patients with IBS should follow as liberal a diet as possible over the long run (McKenzie et al. 2016). Therefore knowledge on the benefits of eliminating even a very limited number of key triggers, such as rye bread, might be of major practical relevance to these patients.

Another strength of the present studies is that the loss of participants was relatively low. All participants in the wheat study and 85% of participants in a 4-week rye study were retained throughout the study period.

Generally, the cross-over designs used in studies I and IV can be conducted with smaller numbers of participants while still detecting statistical significant differences between treatments because participants serve as their own controls. Cross-over studies also mitigate some risks inherent to parallel group studies: randomisation may sometimes fail in such a way that the treatments groups differ already at baseline. In fact, the randomisation could have succeeded better in study II, i.e. the only parallel group study included in this thesis work, i.e. the participants in the sourdough wheat bread group seemed to be slightly more sensitive to wheat than the participants in the yeast bread group. In cross-over studies, it is essential to have a long enough wash-out period between treatment periods in order to ensure that the effect of the first treatment has been totally eliminated before initiating any new treatment period. The present wash-out period was at least four weeks in studies I and IV and this can be considered long enough. No carry-over effect was detected for any end points other than intestinal cramps.

One limitation of the present studies is that we were not able to quantify the FODMAP content of the background diet. Finnish food databases do not contain FODMAP data, especially data on the concentrations of polyols, GOS, FOS and inulin is lacking. Currently, an estimation of FODMAP content of diets in Finland is a practical challenge and was therefore not performed. There is a need for database containing FODMAP data here in Finland whereas in Australia and UK such databases already exist (Halmos et al. 2014, Staudacher et al. 2017).

It is likely that the most sensitive persons were excluded from the 4-week rye study because a willingness to eat rye for the treatment periods was one of the inclusion criteria. Many participants may have thought this was too long to expose themselves to symptoms. Therefore, our results cannot be extrapolated to those who consider that they are very sensitive to rye. In addition, persons with constipation predominant IBS (IS-C) were excluded and consequently the results of the 4-week rye study cannot be extrapolated to persons with IBS-C. The patient population was overwhelmingly female dominant, and therefore the results cannot be certainly extrapolated to males suffering from IBS. Depression or anxiety were not exclusion criteria and therefore the results are likely to be relevant also for people with depression or anxiety co-morbidities.

The number of participants was low in study II, two participants discontinued the study prematurely compounding this limitation. Another point to be taken into account is that hydrogen excretion tests were performed at home by the participants themselves; this may possibly introduce some bias. The power of the study was calculated using hydrogen excretion as the primary end point. In retrospect, it would have been better to use symptoms as a basis for power calculation as they are more clinically relevant. The wheat study did not include any power calculation, as it was a pilot study and no benchmark clinical studies on ATIs existed. Because the number of patients in the wheat study was low, all of the emerging findings need to be interpreted with caution.

The wheat study had a duration of seven days and it is possible that this is not enough for triggering inflammation or attenuating a nocebo effect. However, it is extremely difficult to expose people in long-term studies to foods that they think may be triggering symptoms. Adherence to treatment commonly suffers as the exposure time increases. Balancing the requirements of a long enough treatment period and ensuring adherence in dietary interventions remains a challenge in IBS studies.

It was not possible to analyse the bioactivity of ATIs present in sourdough/yeast bread and this should be done in the future. Furthermore, the amino acid sequences of the ATIs present in the breads were not analysed, and therefore one cannot be 100% sure to which ATI groups the compounds present in the sourdough and yeast bread belong. More studies on different methods will be needed to verify the actual ATI content in wheat breads.

6.3 Perspectives for future research

The present studies and the literature search conducted in this doctoral dissertation provide guidance for new ways of conducting research into IBS and non-coeliac wheat sensitivity. First, the studies should be long enough (i.e. three months or more) whenever possible. Nocebo and placebo effects introduce background noise into the studies that only diminish with time; they may also mask true but more subtle beneficial effects.

It would be interesting to investigate the effect of a low FODMAP rye bread as a part of a holistic low FODMAP diet since here its effects were measured in isolation, and the habitual background diet was continued. Given the effects of low FODMAP diet on microbiota and the restrictive nature of the diet, it would be valuable to examine if the tolerance to FODMAPs and the abundance of beneficial bacteria in intestine could be improved by achieving a slow incremental increase in the levels of fructans/GOS in patients who are adhering to a low FODMAP diet. Here, the intake of FODMAPs increased rapidly by adding regular rye bread to the diet (control diet) but there were no signs of a reduction of symptoms as a function of time (time-effect) during the four week period (results not published). It is possible that a gradual incremental increase in the FODMAP intake might act differently. Administering GOS with increasing doses as a function of time has been claimed to improve its tolerability due to colonic adaptation (Mego et al. 2017). In parallel, the abundance of bifidobacteria increased. Furthermore, Azpiroz et al. (2017) investigated subjects with gas-related complaints who were fed either 8 g/d of inulin or

placebo (maltodextrin, 8 g/d); it was found that exposure to inulin for four weeks decreased intestinal gas retention during the gas challenge test by 22%. To conclude, the available data suggests that the tolerance to FODMAPs can be improved if fructans or GOS are eaten regularly. It is not known if the opposite can also occur; i.e. if a low FODMAP diet further reduces the tolerance of dietary fructans or GOS due to colonic adaptation.

It would be valuable to elucidate the differences in the ATI contents of fermented and non-fermented grain products and verify the results with *in vitro* bio-activation tests which were not performed here. DNA sequencing of ATI molecules are also recommended in the future studies to confirm the nature of the ATIs. From an academic perspective, a direct comparison between ATIs and FODMAPs, could provide direct answers to one crucial question: is it ATIs or FODMAPs that are the causal factors in inducing symptoms? Such studies might be very difficult to arrange because it is far from straightforward to extract ATIs from grains, and there are no commercially available techniques. These limitations make it difficult to determine the dose that should be used in such studies.

An interesting area of research would be to determine the characteristics of the polyphenols and their role in IBS. Polyphenols seem to be metabolised by intestinal microbiota and produce short chain acids with anti-inflammatory properties (Parkar et al. 2013), they may confer beneficial effects on the microbiota (Dueñas et al. 2015) and indeed they have been claimed to decrease intestinal inflammation in experimental studies (González et al. 2011). As discussed previously, the current literature demonstrates that a low-grade inflammation is present, at least in a subset of patients (Simrén et al. 2013). These concepts raise the possibility that an "anti-inflammatory diet" incorporating a high intake of polyphenols might reduce symptoms in irritable bowel syndrome, but no such studies currently exist (Minihane et al. 2015).

Finally, more studies are warranted that would scrutinise the combined role of different substances present in grains. Rather than conducting direct comparisons with one substance against another substance, more studies are needed to evaluate the role of one substance class, such as pure fructans vs. mixtures of substances, such as fructan, ATI, AXOS and gluten.

7 CONCLUSIONS

On the basis of the results emerging from this thesis, the following conclusions can be drawn:

1. Flatulence, rumbling, intestinal cramps and abdominal pain decreased along with colonic fermentation during the consumption of low FODMAP rye bread compared to the consumption of regular rye bread. Nevertheless, no difference was found in overall symptom control as measured by IBS-SSS.

2. Sourdough baking method cleaves the ATIs present in wheat flour more effectively than yeast fermentation, and produces a bread containing only monomeric ATIs. However, cleavage of ATIs did not improve the gastrointestinal tolerance of sourdough wheat bread as compared to regular yeast-leavened wheat bread.

3. No difference was found in pH, transit times or intracolonic pressure between the low FODMAP and regular rye bread periods but the lack of differences might be partly explained by the long time that the SmartPill® remained in the stomach.

4. The low FODMAP rye bread reduced the abundance of *Klebsiella* in fecal samples in comparison to regular rye bread but no other statistically significant changes in microbiota were found between the bread periods.

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